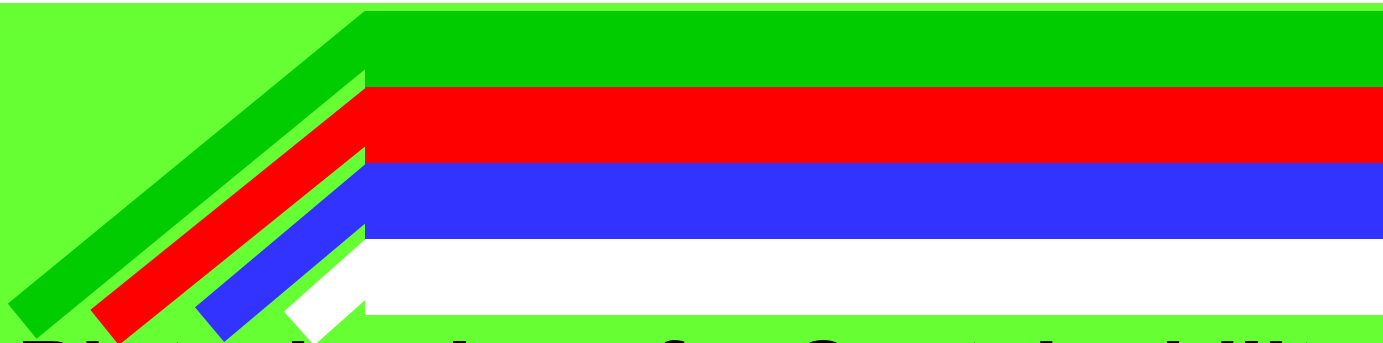




Biotechnology for Sustainability

Achievements, Challenges and Perspectives

Editors
Subhash Bhore,
K. Marimuthu &
M. Ravichandran



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2017

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Cover image: A diagram showing the 17 Sustainable Development Goals (Credit: www.un.org/)

Edited by

Dr. Subhash J. Bhore (Senior Associate Professor)¹,
Dr. K. Marimuthu (Professor)^{1, 2}, and
M. Ravichandran (Senior Professor)^{1, 2}

Address for Correspondence:

¹Department of Biotechnology, Faculty of Applied Sciences, AIMST University, Bedong-Semeling Road, 08100 Bedong, Kedah Darul Aman, Malaysia; Telephone No.: +604 429 8176; e-mail: subhash@aimst.edu.my / subhashbhore@gmail.com

²Chancellery, AIMST University, Bedong-Semeling Road, 08100 Bedong, Kedah Darul Aman, Malaysia; Tel. No.: +604 429 1054 /8103; e-mail: marimuthu@aimst.edu.my / ravichandran@aimst.edu.my

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Dedication

This book is dedicated to all researchers working in various domains of biotechnology and to all stakeholders those are working for the global sustainable development to improve the health of the people and planet.

Preface

World Environment Day (WED) is a biggest global annual event celebrated each year on June 5 to create the positive awareness to preserve the environment and planet earth. This year, the theme for WED-2017 was “Connecting people to nature”. Our environment should be healthy for our growth, development and to achieve the sustainable development goals (SDGs) adopted by the international community to transform the world.

Most recently, António Guterres (United Nations Secretary General) precisely highlighted that “Without a healthy environment we cannot end poverty or build prosperity. We all have a role to play in protecting our only home: we can use less plastic, drive less, waste less food and teach each other to care”. In fact, to achieve the SDGs by protecting environment, everyone needs to do their part.

We strongly believe that biotechnology can play an important role directly or indirectly in achieving various SDGs. Hence, we had decided to publish a book, “Biotechnology for Sustainability” to commemorate the WED and to highlight the achievements, challenges and perspectives in various domains of the biotechnology. In response to our call for articles, we had received 50 manuscripts. The selected articles published in this book are highlighting various issues, achievements, challenges and perspectives for the viable development and sustainability. The World Commission on the Environment and Development defined sustainability as the “development that meets the needs of the present without compromising the ability of future generations to meet their own needs”. The United Nations recent estimate suggest that the world’s food supply needs to be doubled by the year 2050 to keep up with the growing demand. To achieve this is a huge challenge; because, the amount of arable land is continuously decreasing as a result of rising urbanization, saline soils and desertification. Biotechnologists (and plant breeders) around the world are working persistently to produce crops which will boost the food production to meet the growing demand. Genetically engineered crop varieties do offer many promising possibilities to boost nutritive value of the food, sustain farming on marginal lands, and to minimize the loss by creating pests and disease resistant varieties.

The articles published in this book are going to be useful in creating awareness about the environmental issues, natural resources, biodiversity conservation, sustainable development and various biotechnological approaches that could be used to alleviate the respective challenges.

We would like to express our sincere gratitude and thanks to Dato' Seri Utama Dr. S. Samy Vellu, Chancellor and Chairman, AIMST University for his support in publishing this book.

We wish to thank all contributing authors for making a common cause with us. This book publication project could not have been completed without the courteous cooperation of the authors to highlight achievements, challenges and or perspectives in using biotechnological approaches for the sustainability.

We are confident that this book will serve as a reference to various researchers, scientists, academicians and graduate students involved in biodiversity conservation, environmental protection and various fields of biology and biotechnology.

It is hoped that a prudent use of biotechnology in the biodiversity conservation, environmental protection, and production of more and better quality of food, fiber, fuel and drugs will contribute in accomplishing SDGs and to promote peace in the world.

Subhash J. Bhore
K. Marimuthu
M. Ravichandran

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Plant Tissue Culture for Sustainability

C. K. John*

*Plant Tissue Culture Division, CSIR-National Chemical Laboratory, Dr. Homi Bhabha Road, Pune 411008, India; *Correspondence: ck.john@ncl.res.in; Tel.: +91-9822531551*

Abstract: The United Nations has placed great emphasis on sustainability. Three of the most important requirements of sustainable development are: eradicating extreme poverty and hunger, protecting the environment, and conserving biodiversity. Because of human activities the stable functioning of earth's life support system – which includes the atmosphere, oceans, forests, waterways, biodiversity and biogeochemical cycles, is at risk. One of the major contributing factors is the large scale destruction of natural forests. Deforestation had many adverse effects; most importantly, the effects on climate, environment, and biodiversity. The three pillars of sustainable development are: sustainable agriculture, conserving biodiversity, and protecting the environment through reversing the effects of deforestation by large scale afforestation. Plant Tissue Culture can greatly contribute in all the three.

Keywords: Afforestation; biodiversity conservation; micropropagation; plant tissue culture; sustainable agriculture

1. Introduction

The United Nations Summits and Commission Reports from the 1987 Brundtland Commission (World Commission on Environment and Development) report onwards have placed added emphasis on sustainability of all development efforts. Three of the most important requirements are: 1. Eradicating extreme poverty and hunger, 2. Protecting the environment, and 3. Conserving biodiversity. To eradicate extreme poverty and hunger two things are essential: first, sustainable agriculture which makes food available/affordable and second, creation of jobs which translates to purchasing power. One of the major factors in protecting the environment is reversing the loss of natural forests. Conserving biodiversity is of great relevance now than ever before for the reason that our world is fast changing. To have crop varieties suitable for this changing environment is to preserve as much natural

variation in plant varieties as possible. Plant tissue culture can contribute to all the three. In this paper I will elaborate on how Plant Tissue Culture, my area of research, can contribute to Sustainable Agriculture, Protecting Forests, and Conserving Biodiversity.

2. Sustainable development

In 1987 it was the Brundtland Commission (World Commission on Environment and Development) report "Our Common Future" which brought the concept of "Sustainable Development" into common use. The World Commission on Environment and Development was set up by the UN General Assembly in 1983. Brundtland Commission Report defined Sustainable Development as "Development that meets the needs of the present without compromising the ability of the future generations to meet their own needs". According to the Brundtland Commission Report, the needs, in particu-

lar the essential needs of the world's poor, to which overriding priority should be given, and the limitations imposed by the State of Technology and Social organization on the Environment's ability to meet present and future needs should be addressed. The Brundtland Commission Report emphasized the need to integrate economic and ecological factors in decision-making at all levels for sustainable development. These factors include, reviving growth, changing quality of growth, meeting essential needs for jobs, food, energy, water and sanitation, ensuring the resource base, reorienting technology and managing risks. In its broadest sense, the strategy for sustainable development aims to promote harmony among people and between human beings and environment.

In 1992, at the Earth Summit (Rio, 1992) there was consensus that environment, and economic and social development cannot be considered in isolation, and in addition to treaties and agreements on climate change, biological diversity, deforestation, and desertification, the Rio Declaration contains fundamental principles on which nations can base their future decisions and policies, considering the environmental implications of socio-economic development.

In 2000 the Millennium Summit of the United Nations, following the adoption of the United Nations Millennium Declaration, established the eight Millennium Development Goals (MDGs) to be achieved by the year 2015. The MDGs are: 1. to eradicate extreme poverty and hunger, 2. to achieve universal primary education, 3. to promote gender equality and empower women, 4. to reduce child mortality, 5. to improve maternal health, 6. to combat HIV/AIDS, malaria, and other diseases, 7. to ensure environmental sustainability, and 8. to develop a global partnership for development. In the present context Goal 7: Ensuring environmental sustainability is very important. Two of the important targets of MDG 7 are: Integrating

the principles of sustainable development into country policies and programs, reversing loss of environmental resources, and reducing biodiversity loss.

In 2012, the United Nations Rio+20 summit in Brazil committed governments to create a set of "Sustainable Development Goals" (SDGs). On September 25th 2015, countries adopted a set of goals to end poverty, protect the planet, and ensure prosperity for all as part of a 2030 Sustainable Development Agenda. Each goal has specific targets to be achieved in 15 years. The 17 Sustainable Development Goals (SDGs), otherwise known as the Global Goals, are a universal call for action to end poverty, protect the planet and ensure that all people enjoy peace and prosperity always. The goals are interconnected. The key to success on one will involve tackling issues associated with another. The SDGs work in the spirit of partnership and pragmatism, to make the right choices now to improve life, in a sustainable way, for future generations. They provide clear guidelines and targets for all countries to adopt in accordance with their own priorities and the environmental challenges of the world at large. The SDGs are an inclusive agenda. They tackle the root causes of poverty and unite all nations together to make a positive change for both people and planet (UNDP). The 15th SDG of UN relates to Life on land, and involves protecting, restoring and promoting sustainable use of terrestrial ecosystems, sustainably managing forests, combating desertification, and halting and reversing land degradation and halting biodiversity loss.

The stable functioning of Earth's life support system – which includes the atmosphere, oceans, forests, waterways, biodiversity and biogeochemical cycles, is a prerequisite for future human development. However, as per recent research findings this functioning is at risk (Rockström *et al.*, 2009). Further human pressure may lead to large-scale, abrupt, and

potentially irreversible changes to Earth's life support system (Lenton 2011; Barnosky *et al.*, 2012). Likely impacts on humanity include: diminishing food production, water shortages, extreme weather, ocean acidification, deteriorating ecosystems, and sea-level rise. In this backdrop Griggs *et al.* (2013) suggested that we redefine sustainable development as "Development that meets the needs of the present while safeguarding Earth's life-support system, on which the welfare of current and future generations depends." Without economic, technological, and societal transformations, chances of large-scale humanitarian crises exist. Such crises could undermine any gains made by meeting the MDGs. A re-evaluation of the relationship between people and planet is necessary (Griggs *et al.*, 2014).

3. Three pillars of sustainable development

In the second half of the 20th century there was intensification of agriculture in most parts of the world. Intensive agriculture involved: (i) expanding farm lands, by removing natural forests, (ii) better irrigation, by constructing big dams, which again submerged vast forests in their catchment areas (iii) use of chemical fertilizers and pesticides, to produce high yields. Destruction of natural forests had many adverse effects; most importantly, the effects on climate, environment, and biodiversity. Extensive use of chemical fertilizers and pesticides also had their own adverse effects. Excessive use of chemical fertilizers has resulted in nitrate accumulation, increased soil salinity, and water eutrophication. High use of pesticides has resulted in development of resistance in many pest species. In recent years there is much concern about environmental contamination by fertilizers and pesticides.

Sustainable Development, that meets the needs of the present while safeguarding Earth's life-support system, on which the welfare of current and future

generations depends, stands on three pillars:

- i. Sustainable agriculture
- ii. Conserving biodiversity
- iii. Protecting the environment

Increasing food production must involve, developing/ introducing better (efficient, high yielding, insect-pest resistant) varieties of crop plants, conserving biodiversity, and protecting environment. Plant Tissue Culture can greatly contribute in all these.

4. Plant tissue culture

Plant tissue culture is the *aseptic growing of whole plants or parts (cells, tissues/ organs) in/ on defined (synthetic) nutrient media under controlled (environmental) conditions (temperature, light, humidity)*. Usually in glass vessels (test tubes, conical flasks, jam bottles etc.) - for a review see John *et al.* (1997).

Plant tissue culture is based on cellular 'totipotency', the inherent potential of a plant cell to regenerate a whole plant. Unlike animal cells, most plant cells retain the capacity to regenerate the whole organism even after undergoing the final differentiation. In plants, as long as the cells have an intact membrane system and a viable nucleus, even highly mature and differentiated cells retain the ability to regenerate to a meristematic state. Though initially, in the first two decades of the 20th Century progress was slow, standardization of universal plant tissue culture media - White's (White, 1933), Gamborg's (Gamborg *et al.*, 1975) and MS (Murashige and Skoog, 1963) changed the scene. Plant tissue culture media contain minerals, growth factors and a carbon source (usually sucrose). Controlled environmental factors are light (intensity and length – photoperiod), temperature, relative humidity. On/ in a custom standardized medium, and controlled environmental conditions, the explant (starting plant material) - usually young,

undifferentiated tissue, regenerate into whole plants.

4.1. Types of cultures

Different types of cultures are possible: (i) culture of whole plants, (ii) embryo culture (embryo rescue), (iii) organ culture (shoot tip culture, root culture, leaf culture, anther culture etc.), (iv) callus culture, (v) cell suspension and single cell culture, (vi) protoplast culture.

4.2. Callus culture

Callus is an amorphous mass produced by cell proliferation, occurring in an unorganized manner. In nature it is a wound response, or a plant reaction to the presence of micro-organisms, insects, or to some kind of stress. Under *in vitro* conditions callusing is a response to endogenous or exogenous growth regulators. The potential for callus formation is dependent on the tissue (explant) type. Meristematic tissues are more suitable for callus induction than mature tissues. Callus cultures can be maintained for long by sub-culturing the primary callus (callus established originally from the explant), at periodic intervals.

4.3. Somaclonal variations

Long term callus cultures can however, suffer from spontaneously arising genetic variations, reflected in the phenotype of plants regenerated from such calli. These variations are known as *somaclonal variations*. *Somaclonal variations* are reported in many species. The basis of somaclonal variations is not well understood. Chromosomal rearrangements, activation of endogenous transposons, and changes in the status of DNA methylation, are considered to be the contributing factors.

4.4. Suspension cultures

Culture of unorganized plant cells, as single cells/ cell aggregates, in liquid medium. Friable callus when cultured in agitated liquid medium, the cells separate and form a suspension of single cells/ ag-

gregates of few cells. These cells/ cell aggregates grow/ divide/ separate as a result of agitation, and can be continually maintained in this state. Growth of cells in suspension culture can be more easily manipulated in liquid medium than on semi-solid medium. Slowly agitating the liquid medium on a rotary shaker is necessary for the growth of the cultures, which can be sub-cultured. Growth in single isolated cells can be induced by culturing them in hanging drops in micro-chambers. Suspension cultures are useful in plant production by somatic embryogenesis from single cells. In regeneration of plants from callus established on semi-solid media from small cell aggregates, and for the production of secondary metabolites. Suspension cultures can also be initiated from tissue other than callus (Geile and Wagner, 1980).

4.5. Protoplast cultures

Protoplasts are plant cells without cell walls. In 1882, Klercker isolated protoplasts mechanically for the first time. The yield of protoplasts was very low. In 1960, Cocking using enzymes for the first time could isolate protoplasts in large numbers. Protoplasts can be isolated from different plant parts, or from tissues already in culture. Enzymatic isolation is now the most commonly used method. A combination of these two can also be used.

One of the important applications of protoplasts is in somatic hybridization. Many agents like NaNO_3 (Power *et al.*, 1990), a higher pH, and a higher concentration of calcium ions in the medium (Melchers and Labib, 1974), polyethylene glycol (Kao and Michayuluk, 1974; Wallin *et al.*, 1974), and a high strength electric field (Zimmermann and Scheurich, 1981), are used for obtaining fusion between protoplasts. Protoplasts, when placed in appropriate media regenerate cell walls and form calli, from which plants can be regenerated. Protoplasts are used for producing somatic hybrids (para-sexual hybrids), for genetic manipulation,

and for basic studies on a variety of aspects. Regenerating plants from protoplasts is difficult in some species. Conventional hybridization depends on affinity of gametes. Wide crosses are not possible because of well-established cross breeding barriers. Protoplast fusion makes such hybridizations possible.

4.6. Anther (isolated microspore) cultures

Guha and Maheshwari (1964) obtained haploid embryos, directly from anther cultures of *Datura innoxia*. The origin of these embryos was traced to the pollen grains. The potential of anther culture for obtaining haploid plants, and from them by chromosome doubling of homozygous diploid plants was apparent. In 1974, Nitsch had reported regeneration of haploids and homozygous diploids by chromosome doubling, from isolated microspore culture (Nitsch, 1974a; 1974b). Culturing the microspores along with anther wall is essential for success. In isolated microspores, pollen embryogenesis is induced only rarely.

This technique has great potential in plant breeding. Normally it takes selfing for many generations to obtain homozygosity in parental lines required in breeding programmes. This time can be considerably reduced by haploid culture techniques.

4.7. Meristem culture and shoot tip culture

When growing points (meristems) of shoots are cultured they continue their organized growth. The shoots/multiple shoots produced can be rooted to produce plantlets. This capacity has practical application and economic significance for plant propagation.

Culture of the meristemic zones /extreme shoot tip is known as *meristem culture*, and culture of small segments (5-10 mm in size) from the shoot tip is known as *shoot tip culture*. It was known that meristems of virus infected roots are free of the pathogen (White, 1933; 1934). Limasset and Cornuet (1949) found that

shoot tips of virus infected plants are also virus-free. Morel and Martin (1950; 1955) could produce healthy plants from virus-free plants through shoot-tip culture from infected mother plants. This is possible because the pathogen concentration is not uniform in the infected plants, and apical buds of rapidly growing shoots are often not invaded by the virus. Morel (1960) used shoot apices of orchids to obtain their rapid clonal multiplication. Shoot tip culture has two important practical applications: (i) virus eradication and (ii) micro-propagation. These developments were followed by *in vitro* propagation of plants from shoot tip culture. Initially most of the species micropropagated were herbaceous (Morel, 1964; Murashige, 1974). Now methods are available for the micropropagation of a large number of species belonging to a wide range of plant groups.

4.8. Embryo culture

Very young to mature embryos can be cultured *in vitro*. Embryo culture is one of the oldest applications of plant tissue culture in plant breeding. It has many practical applications, and very useful in obtaining hybrid plants from crosses in which post-zygotic incompatibility exists. In post zygotic incompatibility, fertilization and zygote formation occur on cross pollination. The zygote grows, but is not accepted by the endosperm. This results in embryo abortion at some stage of development before maturing of the seed. In such instances when the ovary/ ovule/ embryo with a part of the maternal tissue is excised and cultured on a suitable medium and under optimum culture conditions, it matures to produce a seedling. This procedure is hence called *embryo rescue*. Sharma *et al.* (1980) obtained few hybrids between *Solanum melongena* and *S. khasianum* by this method. Embryo culture is useful also in overcoming seed dormancy and for obtaining seed germination in some vegetatively propagating species in which seeds are produced but

normally do not germinate (e.g. some wild bananas).

4.9. *Invitro pollination and fertilization*

Pre-zygotic incompatibility is one of the major limitations in obtaining hybridization between many plant species and varieties. In pre-zygotic incompatibility, the zygote is not formed on cross pollination. The pollen either do not germinate on the stigma of the female parent or the pollen tube gets arrested at some point of its growth on the stigma/ in the style.

A variety of methods used *in vivo* to overcome this barrier.

Kanta *et al.* (1962) developed an *in vitro* technique for overcoming pre-zygotic incompatibility. In this method, the mature/nearly mature ovaries/ovules are cultured on suitable media and pollinated *in vitro* with cross pollen to obtain hybrids (Zenktler, 1980; Raghavan, 1990).

4.10. *Root cultures*

Tip portions from primary and secondary roots of many plants can be cultured. In 1922, Kotte and Robbins independently postulated that true *in vitro* cultures could be raised from meristematic cells from root tips and shoot tips. Kotte (1922) could cultivate root tips of pea and maize in nutrient media for long, but no sub-culturing were done. Robbins (1922) could subculture his maize root cultures. White (1934) obtained unlimited growth of tomato roots, using the same medium as Robbins (1922), with yeast extract. Root cultures are useful in: (i) secondary metabolite production, and (ii) in basic studies on nematode infections, mycorrhizal associations, and root nodulation by *Rhizobium* bacteria.

4.11. *Organogenesis*

Organogenesis is the process of initiation and development of a structure that shows natural organ form and/or function. It is the ability of non-meristematic plant tissues to form various organs *de novo*; the production of roots,

shoots or leaves. These organs may arise out of pre-existing meristems or out of differentiated cells. Indirect pathway includes a callus stage.

Direct pathway bypasses a callus stage. The cells in the explant act as direct precursors of a new primordium, an organ or a part in its most rudimentary form or stage of development.

4.12. *Somatic embryogenesis*

In plants, embryo-like structures can be generated from non-germ cells (somatic cells), by circumventing the process of normal fertilization. As somatic embryos are formed without fertilization event, they are genetically identical to the parent tissue, and are therefore clones.

Somatic embryogenesis may be direct or indirect. Indirect somatic embryogenesis involves a callus phase prior to embryo production. Direct somatic embryogenesis involves production of embryos from organized tissue without an intervening callus phase. Irrespective of the mode of production, anatomical and physiological features of somatic embryos are highly comparable to zygotic embryos. The morphological and temporal developments of somatic embryos are very similar to that of zygotic embryos. They both proceed through a series of distinct stages, namely, globular, heart, torpedo and cotyledon or plantlet stages for dicotyledons, and globular, elongated, scutellar and coleoptilar stages for monocotyledons. These stages typically span a period of several days. In dicots initially small globular embryos form which undergo isodiametric growth and establish bilateral symmetry. In monocots, especially in grasses, the transition from globular stage follows a series of events occurring simultaneously; such as the development of scutellum, initiation of the coleoptilar notch, tissue differentiation with the development of embryogenic vascular system and accumulation of intracellular storage substances. Somatic embryogenesis is used for: large-scale clonal propaga-

tion of elite cultivars, as an alternative to conventional Micropropagation, producing synthetic (artificial) seeds. Indirect somatic embryogenesis (via callus) or secondary embryogenesis is used in gene transfer. Somatic embryogenesis also offers potential model for the study of molecular, regulatory and morphogenetic events in plant embryogenesis.

4.13. Micropropagation

Micropropagation is the tissue culture method of clonal propagation of plants. Plant tissue culture is rapidly becoming a commercial method for propagating difficult-to-propagate plants, new cultivars (selections, hybrids, transgenic), rare/endangered species. Micropropagation is usually achieved by the release (from dormancy), and growth of pre-existing (axillary/ lateral) meristems in the initial culture. This is followed by repeated enhanced formation of axillary shoots by sub-culture on medium supplemented with plant growth regulators. The shoots produced are rooted either *in vitro* or *ex vitro* (out of culture).

There are many advantages of Micropropagation. Shoot production is reliable and consistent. Multiplication rates can be three-fold to eight-fold a month. Plants produced via shoot culture are usually true-to-type and uniform. Allows propagation of rare/ endangered/ hybrid/ induced mutant/ genetically transformed plants. There also are few disadvantages. PGRs do not release apical dominance in all species. There may be a difference in results between juvenile and mature tissue of perennial species; shoot cultures may require a reversion to juvenility. Rooting of the micro-shoots may be difficult. Getting uniform shoot production *in vitro*, which is very important in commercial operations, may not be possible in some instances. The procedure is relatively labor intensive, with high upfront costs to get started.

4.13. 1. Applications of micropropagation

Rapid and large-scale clonal (genetically uniform) propagation of plants (micropropagation) may allow faster production of plants that are slow to propagate *in vivo*.

The time required for bulking-up of new cultivars before they are commercially introduced can be drastically decreased. Storage of germplasm, e.g. Cryopreservation.

4.13.2. The process of micropropagation

- a. A small piece of the plant to be cloned (the explant) is removed from a healthy, well-maintained stock plant and surface sterilized (explant varies with species, but shoot tips, leaves, stem pieces, lateral buds, and young flowers or floral parts are used).
- b. Surface sterilized explants are rinsed with sterile water, and placed aseptically in/ on specially formulated and sterilized medium in culture vessels.
- c. The explant may proliferate directly by enhanced lateral branching, or the tissue may undergo a certain period of unorganized growth (callus) prior to shoot differentiation.
- d. The growth of the cultures is principally determined by the plant growth regulator (PGR) content of the culture medium (the auxin and cytokinin alone or in combination and concentration/s). Most cultures are established within 4 to 12 weeks depending on the species/ cultivar.
- e. A proliferating shoot culture can be sub-cultured to produce divisions which will multiply rapidly.
- f. Rate of multiplication vary and are affected by many factors. Production of thousands, and in some cases millions of plants a year from a single explant has been demonstrated

5. Role of plant tissue culture in sustainable agriculture

Sustainable agriculture requires efficient, biotic and abiotic stress resistant crop varieties. Germplasm collection and

storage, simple crop improvement methods such as selection and bulking by rapid and large scale cloning by micropropagation can be of great use. Plant tissue culture techniques such as: anther culture, dihaploid production, embryo rescue, and *in vitro* pollination and fertilization can be very useful in developing crop varieties through hybridization. Callus culture, cell suspension culture, organogenesis and somatic embryogenesis are essential for crop improvement through transgenics (various genetic engineering techniques).

6. Role of plant tissue culture in conserving biodiversity

6.1. Biodiversity

"Biological diversity means the variability among living organisms from all sources including, inter alia, terrestrial, marine and other aquatic ecosystems and the ecological complexes of which they are part; this includes diversity within species, between species and of ecosystems." - Definition of Biodiversity by CBD.

6.2. Importance of biodiversity

Biodiversity is essential to: (i) ensure the production of food, fibre, fuel, fodder, etc., (ii) maintain other ecosystem services, (iii) allow adaptation to changing conditions - including climate change, and (iv) sustain rural peoples' livelihoods (Convention of Biological Diversity).

6.3. Threats to biodiversity

Biodiversity is under serious threat as a result of human activities. The main dangers worldwide are: (i) Invasion by alien species, (ii) Environmental degradation, (iii) Climate change and global warming, (iv) Urbanization and habitat conversion, (v) Population growth and ever-increasing demand for resources, (vi) Unsustainable over-exploitation of natural resources.

Agriculture contributes significantly to conservation and sustainable use of biodiversity. However, it is also a ma-

jor driver of loss of biodiversity. This puts in jeopardy the sustainability of agriculture and ecosystem services and their ability to adapt to changing conditions. This also poses serious threat to food and livelihood security.

6.4. Plant tissue culture methods for conserving biodiversity

Plant Tissue Culture offers novel options for collection, multiplication and medium/ long-term *ex situ* conservation of plant biodiversity. By plant cell, tissue, and organ culture techniques, rapid and large scale multiplication and season independent production of planting material is possible. This has helped in the conservation of many endangered species. Medium-term conservation is achieved by slow growing cultures. Cryopreservation (at -196°C , in liquid nitrogen) allows the safe and cost-effective long-term conservation.

6.5. *In vitro* collection

Potential advantages of *in vitro* methods are: (i) Less space requirement, (ii) Pathogen-free plants, (iii) No need for transfer (under storage conditions), (iv) Stored cultures can be used as stock for vegetative preservation, and (v) International exchange of plant material made easy because, no use of soil, and no pathogens.

Basic goals of an *in vitro* storage system are: to maintain genetic stability, to keep in indefinite storage without loss of viability, and most importantly, to be economical.

Three types of Plant Tissue Culture systems are available. They are: (i) Normal growth, (ii) Slow growth, and (iii) Cryopreservation.

6.5.1. Normal growth

Normal Growth is achieved either on semi solid media or in liquid media. Normal growth is similar to multiplication stage in micro-propagation, and requires frequent sub-culture. Considered as genetically stable when achieved through di-

rect organogenesis from apical buds / axillary buds as explants.

6.5.2. *Slow growth storage*

By deliberate slowing down of growth cultures can be stored at least for 6 months and maximum up to 6 years without sub-culturing. There are many ways to achieve slow growth. First, by manipulating storage temperature (cold storage at 1-9°C) and light (low light intensity). Second, increasing osmotic potential of the media [by using osmotically active compounds such as sucrose (at higher ~6%), mannitol etc.]. Third, by addition of inhibitors or retardants, for e.g. mineral oil overlay (callus), reduced oxygen tension etc.

Plant Growth Retardants are chemicals that slow cell division and elongation in shoots. They cause plants to be shorter and more compact, interrupt cell division, stem elongation, and inflorescence / flower formation. But roots continue to grow. Plant growth retardants may reduce the natural Gibberellic acid, or may produce more ethylene.

6.5.3. *Cold storage*

Storage at non-freezing temps, from 1-9° C dependent on species. Storage of shoot cultures (stage I or II) works well for strawberries, grapes, may be for many more spp. Transferred to fresh medium every 6 months/ annually/ or longer periods basis. Advantages of cold storage are: (i) simple, (ii) high rates of survival, and (iii) useful in micropropagation (especially in periods of low demand). The disadvantages are: (i) may not be suitable for some tropical, subtropical species because of susceptibility to cold injury, (ii) requires refrigeration, which is more expensive than storage at ultra-low temperatures (in cryopreservation). An alternative to cold storage is the use of a medium with reduced nutrients and lacking sucrose (as reported in coffee).

6.5.4. *Cryopreservation*

Cryopreservation is the storage of living tissues at ultra-low temperatures (-196°C). It is useful in conservation of plant germplasm of vegetatively propagated species, recalcitrant seed species (coconut palm etc.), conservation of tissue with specific characteristics, cell lines producing secondary metabolites, genetically transformed tissues, tissues competent to transformation/ mutagenesis, pathogen (virus) eradicated tissue for future multiplication (as is done in Banana).

Cryopreservation procedures are available only for limited number of plant species. Each species/ variety/ tissue type, needs standardization for: explant size and type, water content, and natural freezing resistance. Most studies on cryopreservation of plants involve only one or a few genotypes. Only few plant germplasm collections stored in liquid nitrogen currently exist (with a relatively limited number of accessions).

7. **Role of plant tissue culture in protecting the environment**

Forests are complex ecosystems, predominantly composed of trees and shrubs, and usually have closed canopies. There is nearly 4 billion hectares of forest in the world (this is about 30% of the total land cover). Depending on the physical, geographical, climatic and ecological factors, there are different types of forests like evergreen forest (mainly composed of evergreen tree species) and deciduous forest (mainly composed of deciduous tree species). India's recorded forest area is 76.52 million hectares. This is 23.28% of the country's total geographical area. Over 90% of the forest area is under government ownership and is managed by the forest departments of the state governments (State of Forests Report, 2009). Forests are important both economically and ecologically, and render many services to the life support system of earth.

Forests are the primary source of wood. Wood is used to fulfil three basic needs: (i) energy, (ii) construction materi-

al, and (iii) industrial raw material. About 2 billion people in the developing world are dependent on forests for their basic energy needs (fuel for cooking food). Until recently wood has been the chief construction material. High strength to weight ratio and availability in many kinds, ease of cutting and shaping with simple tools, and insulation to heat, make wood an ideal construction material for many purposes. Major industrial uses of wood are: (i) paper and pulp, (ii) rayon, and (iii) plywood. Besides wood, forests are a source of a variety of non-wood forest products (NWFPs). Thus forests contribute greatly to the economy. Around 1.6 billion people depend on forests for their livelihood. This includes some 70 million indigenous people.

Forests play an important role in maintaining ecological balance. Forests are atmospheric filters. They are the major suppliers of oxygen. In photosynthesis they fix atmospheric carbon dioxide into carbohydrates, sugars, proteins, and many forms of biomass, thus playing a significant part in the global carbon cycle. Forests contribute large quantities of moisture to the atmosphere, thus regulating climate. Forests also conserve soil and water resources.

The term forest implies 'natural vegetation' of the area, existing from thousands of years and supporting a variety of biodiversity. More than half of the known terrestrial plant and animal species live in forests (Millennium Ecosystem Assessment, 2005). The forest ecosystem has two components - biotic and abiotic. The living component includes plants (trees, shrubs, herbs etc.), animals and microorganisms. Forests are home to more than 80 per cent of all terrestrial species of animals, plants and insects. Globally, deforestation is the major cause of loss of biological diversity, and is a matter of great concern (Laurance, 2007).

Worldwide, the area of natural forests decreases by some 13 million ha annually (this is about 3% of the total forest area). This loss of forest area is mostly

due to conversion to agriculture land and urbanization. Deforestation has severe consequences for the environment and climate. More than 2000 times the total energy consumption of the world population, of solar energy reaches the earth's surface. Because of deforestation, not only this natural source of energy is wasted, but also has a serious negative impact on the environment, by way of surface heating, and desertification.

In the tropical and sub-tropical regions of the world, receiving about 600 mm rainfall and above, plantation forestry of economically important tree species (say teak for timber and eucalypts for pulp) can take away pressure for forestry resources from natural forests and can add to the forest cover. For this, large numbers (in millions) of planting material of superior varieties (fast growing, better adapted, disease and insect-pest resistant etc.) are necessary. But forest tree species are difficult to breed, because of their long generation cycles, highly heterozygous natural populations, openly cross pollinated nature, and lack of knowledge about their genetics.

Clonal propagation of 'superior' genotypes (identified for desirable traits) through tissue culture has been used very profitably in case of many tree species. Eucalypts have been multiplied and used for plantation forestry for a long time (FAO Report, 1981). Eucalyptus wood from plantation forestry has been used as timber, industrial raw material and fuel. Plantation forestry using eucalypts may not be suitable for some places because of high water demand. Teak (*Tectona grandis* Linn. f.) is another success story. In early 1980s, scientists from CSIR-National Chemical Laboratory, Pune for the first time regenerated complete plantlets from an 80 year old 'elite' tree (Gupta *et al.*, 1983). Poplars (*Populus spp.*) are another tree species which clonally propagated through plant tissue culture techniques, and widely cultivated in plantation forestry in many parts of the world. In India, poplars (*Populus spp.*) are the

most popular tree species in agro-forestry production systems. Poplars are usually intercropped with agricultural crops like wheat, rice and sugar cane. Poplars are well known for their fast growth, outstanding properties and quick and high financial returns. Timber from poplars often forms the backbone of match, paper, sports goods, plywood, and composite board industries.

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Traditional Medicine of the Tribes in Tamil Nadu and Its Sustainable Use through Biotechnology

Valli Gurusamy¹, Kavitha Valampuri John², Usha Raja Nanthini Ayyakkannu²,
Ramani Bai Ravichandran^{3,*}

¹Vice Chancellor, Mother Teresa Women's University; ²Department of Biotechnology, Mother Teresa Women's University; ³Department of Zoology, University of Madras, India;

*Correspondence: rramani8@hotmail.com; Tel: +91 9444020828

Abstract: India is a land of mega biodiversity representing about 7% of the world's flora and 6.5 per cent of world's fauna. Tamil Nadu, one of the southern most states of India, is rich in forest cover and cultural diversity. Genomic evidence supports the peopling of Tamil Nadu from the 1st wave of migration of humans from the 'Out of Africa' exodus and points out that the tribes of state were among the earliest settlers in the region. The tribal population of Tamil Nadu represents 1.02% of the total population of the state. Living in close association with the forest, they have accumulated a treasure trove of ethno botanical knowledge in the form of traditional medicine. The future of sustainable use of renewable forest product lies with the molecular tools of Biotechnology. We present here an analysis of the documented literature of the medicinal plants used by the tribes of Tamil Nadu for treatment of common disorders. We also present the challenges and prospects within the scope of Biotechnology to ensure sustainable use of traditional medicine for the betterment of mankind and environment.

Keywords: Biotechnology; sustainable; tribes; traditional medicine; Tamil Nadu.

1. Introduction

India is a land of enormous cultural, linguistic and religious diversity presumably because of Man's long stay, for the past 50-70,000 years in this continent. This is an outcome of various migrations that took place into India, serving as a major corridor for the dispersal of modern humans out of Africa (Cann, 2001). Archaeological evidences indicated that the Indian subcontinent was peopled by various migrations since Palaeolithic (300-400,000 BCE), starting with the Late Pleistocene (Misra, 2001). 'The Castes and Tribes of Southern India' was an attempt to catalogue these populations (Thurston, 1909). The knowledge of the medicinal value of plants, animals and other substances and their uses goes back to the time of the earliest settlers probably

along with their 1st wave of migration out of Africa. Traditional Medicine is the sum total of long-standing information on the knowledge, skills, and health practices based on the theories, beliefs, and experiences indigenous to different cultures or local communities. Traditional medicine incorporates plant, animal and mineral based medicines and encompasses spiritual therapies, manual techniques and exercises which can be applied singularly or in combination for the maintenance of health through the prevention, diagnosis, improvement or treatment of physical and mental illness. Traditional knowledge has been well preserved and orally passed from one generation to the next in the form of stories, legends, folklore, rituals, songs, art, and even laws. Since there is no written script the exchange of know-how between diverse communities is a

process of evolution through trial and error which makes documentation and record-keeping almost impossible. This process of exchange and assimilation is continuous, and today there is a growing awareness among the medical community about the intrinsic value of traditional medicine, and as a result in India Ayurveda, Unani and Siddha have entered the mainstream to compliment biomedicine. Contemporary Indian society faces the challenge of integrating the best of the different healing traditions to provide a holistic health care.

2. Traditional knowledge

Even before classical medical knowledge of ancient India was codified into the canonical texts of Ayurveda in the 6th century BC, there were abundant sources of medical knowhow in the sub-continent from prehistoric times. Traditional healers can be either folk or tribal healers and have worked in intimate relation with their environment. Traditional healing ranges from simple home remedies related to nutrition and treatment for minor illnesses, to more sophisticated procedures such as midwifery, bone setting, blood-letting (therapeutic phlebotomy) and treatment of snake bites and mental disorders. Some healing practices were considered to be sacred and were associated with rituals that helped safeguard them for there is a substantial overlap between healing plants and sacred plants. Categories of traditional healers are traditionally trained healers, old individuals of the community, educated individuals acquiring certain knowledge from their predecessors, ancient inscriptions in the form of copper plate/palm leaf writings, old and recent publications in regional language.

3. Indian ethnobotany

India is one of the richest countries in the world not only in biodiversity but also in different ethnic groups of an-

cient lineage from the 1st wave of the 'out of Africa' exodus and thus in vast ethnobotanical knowledge. According to the 2011 census, 104 million tribal people speaking over 227 linguistic groups inhabit varied geographic and climatic zones of the Indian subcontinent. Ethnomedicine includes plants, animal products and minerals used by tribal communities of a particular region or country for medicinal purposes other than those mentioned in classical streams of the respective cultures. Tribal people have been using a large number of wild plants as documented by ethnobotanical investigations. The application of most of the plants recorded are either lesser known or hitherto unknown to the outside world. India has been the country most concerned about the conservation of its medicinal plants. There are over 45,000 species of vascular plants reported from India of which as many as 15,000 may be used medicinally. The folk medicine system of India use about 5,000 plant species with about 25,000 formulation, whereas the tribal medicine involves the use of over 8,000 plant species with about 1,75,000 specific preparations (Pushpangadan and George, 2010). More than 90% of the raw material for traditional medicine comes from wild harvesting as this the common method used for collecting them (Tandon, 1996; Gupta 1998; Ved *et al.*, 1998). About 71 medicinal plant species are classified as "rare", and of this 92% are in active trade, and 74% are traded nationally. It has been estimated that between 4,000 and 10,000 medicinal plant species in India face extinction in the local, regional and national levels (Hamilton, 2004). In an effort to create leadership in affordable and holistic health care, India is committed to promoting traditional medicines like Ayurveda which remained untapped due to inadequate scientific scrutiny. Steps are being taken to bring in regulatory amendments in research and effective enforcement for integration of quality products, practices and practitioners into the AYUSH (Ayurveda, Yoga and Naturopathy).

thy, Unani, Siddha and Homoeopathy) system at central and state level.

4. Traditional knowledge of the tribes of Tamil Nadu

According to the 2001 census, tribal population in Tamil Nadu is 6, 51,321 which constitute 1.02% of the total population. There are 36 tribes and sub tribes in Tamil Nadu. Out of the 36 Scheduled Tribe communities in the state, about six tribal populations Todas, Kotas, Krumbas, Paniyas, Irulas, Kat-tunayakas have been classified as primitive tribes with incredibly high anthropological significance. The primitive tribes occupy the length and breadth of the Nilgiri district in the Western Ghats of Tamil Nadu. One sixth of the land mass of Tamil Nadu is covered by forests. The tribes of Tamil Nadu live in and around the reserved forests and have gained immense knowledge on the use of forest produce to treat common disorders.

Todas: They are called by other names like Tudas, Thuduvans, and Todar (Kavitha V.J., 2008). They are professional pastoralists and dairy men, a purely pastoral economy in India today, living in the higher altitudes in the traditional houses called “Munds” that are half barrel shaped and are vegetarians. Their dialect is independent form of Dravidian Tamil-Malayalam. They are fair skinned and wear ornaments and their dress is akin to the Roman ‘toga’. They have two exogamous divisions called Tarthar and Teivali. There are five socially distinguishable sects (clans) such as Pelki, Pekkan, Kuttan, Kenna and Jodi (Rajan and Sethuraman, 1993). Their population was 1,600 in the 2001 census.

Kotas: Their other names are Koter, Kothewars, and Kohatur. The Kotas are musicians and excellent craftsmen having mastery over ironworking. They are light skinned, with copper hair. They speak the “Kota” a Dravidian language. Their distribution in the Nilgiri district is confined only to seven villages inhabiting

in moderate altitude of the district namely: New Kotagiri (Aggal), Kil-Kotagiri, Kundah, Kallimalai, Gudalur, Trichigadi and Sholur Kokal. Their settlement is called the “Kokkal” with linear row of houses in streets, ‘Keri’. Each village has three Keri known as Kizhkeri, Nadukeri and Melkeri. Keri, clan, exogamy is noteworthy among Kotas (Kavitha V.J., 2008). Their chief diety is Kambattrayan. Their population was 1,894 in 2001 census.

Irulas: They are also called as Iuvan, Villiar. The Irulas are distributed in the lower altitudes of the Nilgiri hills (district). They are negrotoid in appearance whose chief occupation is as plantation labourers in the estates. Their settlements are called “Aral”. Their dialect is Tamil mixed with Malayalam. Their community is divided into seven exogamous clans (Kuems): Kupper, Sambe, Kalkatti, Kurunagar, Devanan, Peradar and Pungar (Rajan and Sethuraman, 1991). They are basically hunter gatherers. Their population was 6,700 in 2001 census.

Kurumbas: The Kurumbas practice hunting food gathering economy, well-versed in honey collection techniques. They are plain dwelling people living in the interior forests of the district. Their staple foods are wild tubers (*Dioscorea bulbosa*), wild fruits and other minor forest produces. Their settlements are called “Mottam”. They are dark skinned and speak the Kurumba dialect. Kurumbas are a heterogeneous population having divisions such as Halu Kurumbas, Betta Kurumbas, Mullu Kurumbas, Jess kurumbas and Urali Kurumbas. Their population was 6,872 in the 2001 census.

Paniyas: The Paniyas are negrotoid people living in bamboo huts at the junction of Kerala and Tamil Nadu border. They work as labourers with Wayanad Chettis though they were basically hunter gatherers. Their settlements are called “Paddi”. They possess excellent skills in the art of fishing by employing certain plant parts like bark of *Eugenia* sp. and leaves of *Aibizzia* sp. as stupefying

agents. They speak the Paniya dialect, practice Illam, patilineage, and their inheritance is by "Marumakkathayam". They numbered 6,700 in 2001 census.

Kattunayakas: They are also called as Shola Nayakans, Jenu or Teen Kurumbas. They are another group of forest dwellers who are nomadic in nature, their staple foods are honey, wild fruits and tubers. Their settlements are called "Paadi". They are short and black with protruding forehead. They have curly hair and speak Kannada language. Eating bison flesh is a cultural taboo with them. The social customs and religious practices of Kattunayakas are akin to Kurumbas in many respects. Their population was 1,425 in the 2001 census.

Paliyars: They are found in the hilly regions of Madurai, Dindigul, Theni, Tirunelveli and Virudhunagar districts. It is believed that Paliyars are indigenous people of Palani hills of Kodaikanal and speak Tamil. Physically they are similar to the Semong of Malaya and other Indian tribal communities. They can be grouped into three categories based on their life styles, namely, nomadic, semi nomadic and settled. Nomadic Paliyars don't build houses; they live temporarily in rock caves called 'Pudai'.

Ethnobotanical traditional knowledge for the tribes of Tamil Nadu was retrieved using Pubmed and Google using the keywords ethnomedicne, tribes, Tamil Nadu. The ethnobotanical data presented here included knowledge from seven tribal groups of Irula, Paniya, Kurumba, Kota, Thoda, Kattunayakans and Paliyars (Table 1). These published research articles were then analysed manually to ascertain the traditional knowledge of these communities related to the study area and the usage pattern of the medicinal plant species. The data was further analysed using graphical representations for summarizing and interpreting for the major families of plant species represented, part of the plant used and cure for disorder from the traditional me-

dicinal knowledge of the tribes of Tamil Nadu.

5. Ethnomedical wealth of tribes of Tamil Nadu

A total of 229 medicinal plants used by the tribes of Tamil Nadu belonging to 79 families for the treatment of more than 40 disorders were documented. The percent representation of the families of plants used as medicine by the tribes of Tamil Nadu is represented in Figure 1. Euphorbiaceae is the largest family represented by 18 species at 12% followed by Fabaceae by 14 species (9%), Lamaceae by 13 species (8%), Asteraceae by 11 species (7%), Solaneaceae and Rutaceae by 10 species each (6%) and Asclepiadaceae by 8 species (5%). This data marks a direction for scientific researchers as to which of the families to search for to identify bio active compounds.

Of the primary parts of the plant used the leaves formed 40% of usage in traditional medicine followed by the root and bark at 11%, whole plant and fruit at 8%, stem at 7% and seed at 5% (Figure 2). It is of utmost importance to see this data in the light of the major families represented for medicinal use by the tribes of Tamil Nadu. It is also important to test these bioactive compounds from different parts of the plant as a combination and to use bioactive compounds different families in conjunction for any particular disorder.

The data when analysed for major remedies against diseases it revealed that the tribes of Tamil Nadu had traditional remedies for wounds (31) and skin problems (29), followed by stomach aches (17), diarrhoea and headache (13), Cold, cough, fever (12) and rheumatic diseases, gastric disorders and toothache (9). It is interesting to note that the ethnomedicine of the tribals has 9 remedies for women to ease labour pain and 3 to induce lactation (Figure 3). Tribal ethnomedicine also has remedies for diabetes and jaundice (5).

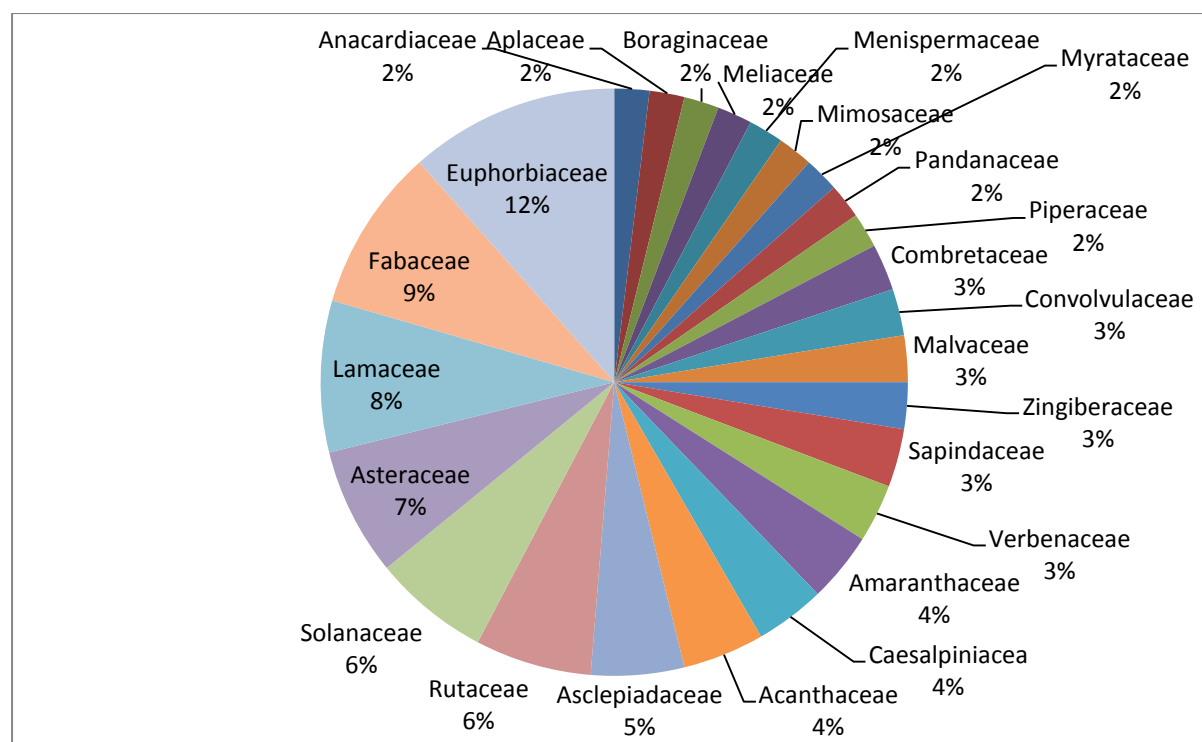


Figure 1: Percent representation of major plant families used by Tamil Nadu tribes as medicine.

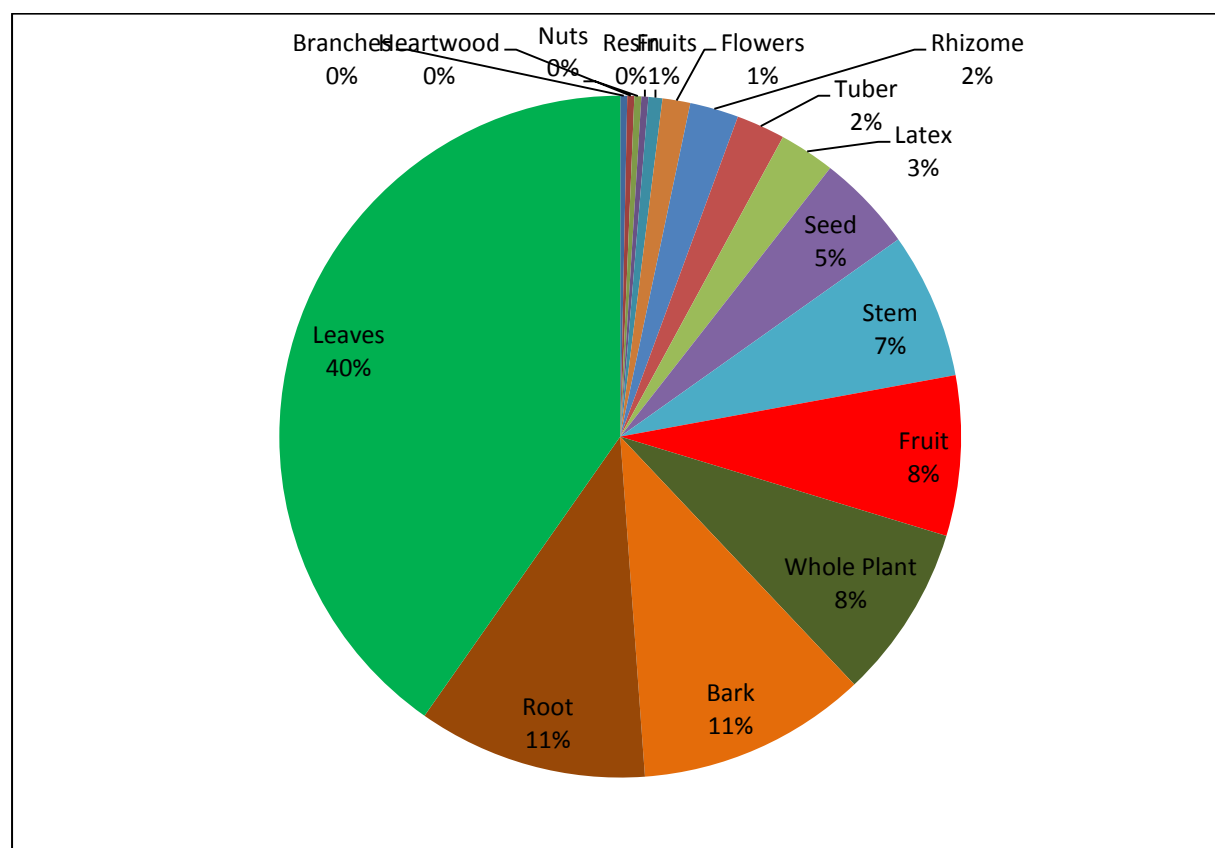


Figure 2: Parts of the Plant used in traditional healing among the tribes of Tamil Nadu.

Table 1: Ethnomedical traditional knowledge of the tribes in Tamil Nadu

No.	Botanical Name	Local name	Part Used	Medicinal uses	Tribe	Reference
Acanthaceae						
1	<i>Adhatoda zeylanica</i> Medicus	Adathodai	Leaves	Asthma, Cold, fever	PL	e
	<i>Adhatoda zeylanica</i> Medicus	Auduthoda	Root, bark, flowers	Cough, asthma, eye pain	IR	g
2	<i>Andrographis lineata</i> Wallich ex Nees	Siriyangai	Leaves	Cough, diabetes, scorpion and snake bite	PL	e
3	<i>Andrographis paniculata</i> (Burm.f.) Wall. ex Nees	Periyangai or Nilavembu	Leaves	Scorpion sting and snakebites, menorrhagia	PL	e
4	<i>Asystasia gangetica</i>	Valukai keerai	Leaves	Appetite	PL	e
5	<i>Blepharis maderaspatensis</i> (L.) Roth.	Vettukaaya pachilai	Leaves	Fractured bones, Cuts	PL	e
6	<i>Phlebophyllus kunthianum</i> Nees	Kurinji chedi	Leaves	Nervous disorder	PL	e
Alangiaceae						
7	<i>Alangium salvifolium</i> (L.f.) Wangerin	Alinji	Fruit	Eye infections	PL	e
Amaranthaceae						
8	<i>Achyranthes aspera</i> L.	Uthruk	Leaves	Cuts, wounds and sores	KO	b
	<i>Achyranthes aspera</i> L.	Cherukadalai	Whole Plant	Sprain ached in the Joints	KT	b
	<i>Achyranthes aspera</i> L.	Nayuruvi	Leaves	Diverticulosis & Diverticulitis	KU	i
	<i>Achyranthes aspera</i> L.	Nayurvi Geeda	Whole Plant	Ease child birth and labour pain	KU	b
	<i>Achyranthes aspera</i> L.		leaves	New born babies, lactation	IR	g
9	<i>Achyranthes bidentata</i>	Blume, Kithoop	Leaves	Rapid healing of wounds	KU	i
10	<i>Achyranthus bidentata</i> Blume	Naiyur	Leaves	Skin disorders including scabies	KO	a
11	<i>Aerva lanata</i> (L.) Juss.ex Schult	Kannupila & Pan-naipoo	leaves	New born babies, lactation	IR	g
	<i>Celosia argentea</i> L.					
12	<i>Alternanthera sessilis</i> (L.)	Nilakirai	Leaves	Diarrhoea	KU	i
	<i>Alternanthera sessilis</i> (L.)	Nilakirai	Leaves	Roughage	KU	i
13	<i>Amaranthus gangeticus</i> L.	Mulai keerai	Whole plant	Good digestion, Constipation,	KU	i

Table 1: Continued...

Anacardiaceae						
14	<i>Anacardium occidentale</i>		Bark, Fruit latex	Fever, warts	IR, PA	c
15	<i>Mangifera indica</i>	Maavae pattae	Bark	Stomach pain	IR, PA	c
16	<i>Spondias pinnata</i>	Kattu Maa	Bark	Acute diarrhoea	IR, PA	c
Annonaceae						
17	<i>Annona squamosa</i> L.	Seetha mara	Seeds	Vermifuge	PA	b
Apiaceae						
18	<i>Centella asiatica</i> (L) Urban	Vallarai	Whole Plant	Refrigerant	TH	b
	<i>Centella asiatica</i> (L) Urban	Gottala	Whole Plant	Toothache	KT	b
	<i>Centella asiatica</i> (Linn.) Urban.	Kidth Kot	Leaves	Stomach problems Cools the body	KO	a
19	<i>Coriandrum sativum</i> Linn.	Kothumull	Leaves	Refrigerant and diuretic	KO	a
Aplaceae						
20	<i>Buplerum wightii</i> P.K. Mukherjee,	Malai seragam	Root	Easy delivery	IR-S	d
21	<i>Centella asiatica</i> (L) Urban,	Kutheraikokku	Leaves	Digestive agent, blood circulation	IR-S	d
22	<i>Heracleum ceylanicum</i> Gardner ex C.B. Clarke	Poonaikal sedi	Leaves	Insect allergy	IR-S	d
Apocymeeae						
23	<i>Rauvolfia serpentina</i> (L.) Benth. ex Kurz	Chivanamelpodi	Root	Stomachache	KT	f
24	<i>Alstonia scholaris</i> (L.) R.Br.	Paalooram pattai	Stem	Lactation	PL	e
Araceae						
25	<i>Acorus calamus</i> L.	Vasambu	Rhizome	Speech	PL	e
26	<i>Colocasia esculenta</i> (L.) Schott	Kattu shembu	Tuber	Worms	KT	f
	<i>Colocasia esculenta</i> (L.) Schott.	Chembu	Leaves & Rhizome	Small red colour boils appearing on the skin	KU	h
Arecaceae						

Table 1: Continued...

27	<i>Phoenix sylvestris</i> (L.) Roxb.	Injai	Stem	Easy Delivery	IR	g
Aristolochiaceae						
28	<i>Aristolochia indica</i> L.	Perumanthikodi	Root	Fever	IR	g
29	<i>Aristolochia tagala</i> Cham.	Modhalaikodi.	Leaves	Diarrhea and vomiting	IR-S	d
Asclepiadaceae						
30	<i>Ceropegia candelabrum</i> L.	Perun kodi	Leaves	Headache	PL	e
31	<i>Cryptolepis buchananii</i> Roem & Schul	Paalkodi/Karunkodi	Latex	Wound	PL	e
32	<i>Gymnema hirsutum</i> W&A	Sakarasedi	Leaves	Diabetes	KU	i
33	<i>Gymnema sylvestre</i> (Retz.) R. Br. Ex	Sirukurinjan	Leaves	Diabetes and nervous disorder	PL	e
	<i>Gymnema sylvestre</i> R.Br.	Sirukurinjan & ha-karikolli	Leaves	Diabetes	IR	g
34	<i>Hemidesmus indicus</i> H.f.	Nannari	Whole Plant, Root, leaves	Fever, menorrhagia, stomach-ache	PL	e
35	<i>Holostemma ada-kodien</i> Schult.	Ada kizhangu	Tuber	Fever	KT	f
36	<i>Pergularia daemia</i> (Fors.) Chio	Veli parutthi	Leaves	Headache	PL	e
37	<i>Tylophora indica</i> (Burm. f.) Merr.	Nangilai	Leaves, Root	Snakebite	PL	e
Asparagaceae						
38	<i>Asparagus racemosus</i> Willd.	Ammaikodi	Tuber	Stomachache	KT	f
Asteraceae						
39	<i>Adenostemma lavenia</i> (L.) Kuntze	Kasirukai	Leaves	Skin diseases	IR-S	d
40	<i>Ageratum conyzoides</i> L.	Nasar soppu	Leaves	Cough and cold	KU	b
	<i>Ageratum conyzoides</i> Linn.	Pugudu thalai	Leaves	Wound	KO	a
41	<i>Artemisia nilagarica</i> (C. B Clarke) Pamp.	Manikoland	Leaves & stem	Removing worms from wounds both in humans and animals	KU	i
42	<i>Artemisia parviflora</i> Buch – ham. Ex Roxb.	Railpundu	Leaves	Headache	IR-S	d
43	<i>Bidens pilosa</i> L.	Katu kunni	Leaves	White patches on the legs	KU	h

Table 1: Continued...

44	<i>Blumea lacera</i> (Burm.f.) DC.	Navakkarandai	Leaves	Improving vision	KT	f
45	<i>Chromolaena odorata</i> (L.) King & Robins.	Vettukkaya pacha	Leaves	Cuts and wounds	KT	f
46	<i>Elephantopus scaber</i> L.	Anashovadi	Root	Stomach ache	KT	f
47	<i>Siegesbeckia orientalis</i> Linn.	Potaz	Leaves	Skin rashes, insect bites and allergies	KO	a
	<i>Sigesbeckia orientalis</i> L.	Nadukadachi	Leaves	Wounds and parasitic skin problems	KU	h
48	<i>Sonchus oleraceus</i> L.	Kaalaadi pachilai	Leaves	Wound	PL	e
49	<i>Tridax procumbens</i> L.	Vettukayapoondur	leaves	Wounds to stop bleeding	IR	g
	<i>Tridax procumbens</i> L.	Vettukkaya thalai	Leaves	Sores	KT	f
Balanophoraceae						
50	<i>Balanophora fungosa</i> Fors and Fors.	Vaer chedi	Whole Plant	Skin disease	PL	e
Berberidaceae						
51	<i>Mahonia leschenaultii</i> (Wight & Arn.) Tak. ex Gamble	Mullu kadambu	Bark	Skin disease	PL	e
52	<i>Berberis tinctoria</i> Lesch	Jakkala	Leaves & stem	Dysentery, Bloating of stomach	KU	i
Bignoniaceae						
53	<i>Radermackeria xylocarpa</i> (Roxb.) K. Schum.	Vadencarni	Stem	Fever	KT	f
Bischofiaceae						
54	<i>Bischofia javanica</i> Blume.	Romaviruksha patta	Bark	Nervous disorder, hair growth	PL	e
Boraginaceae						
55	<i>Carmona retusa</i> (Vahl) Masam.	Kurangu vetthilai	Leaves	Fertility	PL	e
56	<i>Nasturtium indicum</i> (L.) DC.	Kadge	Root Portion	Ear diseases	KU	i
57	<i>Trichodesma zeylanica</i> R.Br	Jalke maram	Root	Round patches appearing on the skin	KU	h
Burseraceae						
58	<i>Boswellia serrata</i> Roxb. Ex Colebr.	Kungiliyam	Resin	Cold	PL	e

Table 1: Continued...

Caesalpiniaceae						
59	<i>Caesalipinia bonduc</i> (L) Roxb.	Porumaielai	Root, seeds	Gastric disorders Increase body weight	IR-S	d
60	<i>Bauhinia racemosa</i>	Mara avara	Bark	Boils	IR, PA	c
61	<i>Cassia auriculata</i> L.	Avarai	Fruit, leaves, flowers	Skin and scalp	IR	g
62	<i>Cassia fistula</i>	Gaggai pattai, Konnai mara	Bark	Sudden “sicknesses” , diarrhoea and stomach pain	IR, PA	c
63	<i>Cassia fistula</i> L.	Konnei	Stem bark	Stomach ache	KT	f
63	<i>Pterolobium hexapetalum</i>	Kari indu	Leaves	Ease delivery pain	PL	e
64	<i>Tamarindus indica</i>	Puli	Fruit	Nursing mothers, eczema	IR, PA	c
Caparidaceae						
65	<i>Capparis sepiaria</i> L.	Thoratti	Root	Wounds and scratches	IR	g
	<i>Capparis zeylanica</i> L.	Adandai	leaves	Increase appetite	IR	g
66	<i>Celome monophylla</i> L.	Kadugu sedi	Leaves	Earache	IR-S	d
Caricaceae						
67	<i>Carica papaya</i> L. Poppilli	Poppilli mara	Fruit	Indigestion and Constipation	KU	i
Caryophyllaceae						
68	<i>Drymaria cordata</i> (L.) Roem. & Schult.	Kodi charai	Leaves	Heel cracks	PL	e
Celastraceae						
69	<i>Celastrus paniculatus</i> Willd.	Valulurai	Root	Body pain	KT	f
Chenopodiaceae						
70	<i>Chenopodium ambrosioides</i> L.	Jaregida	Whole plant	Intestinal cramps	KU	h
Colchicaceae						
71	<i>Gloriosa superba</i> L.	Kodanki kizhangu.	Tuber	Sleeping tablet	IR-S	d
Combretaceae						

Table 1: Continued...

72	<i>Pterocarpus marsupium</i> Roxb.	Vengai	Stem	Rheumatic pain	KT	f
73	<i>Terminalia bellirica</i> (Gaertn.) Roxb.	Tani	Bark	Body pain	KT	f
74	<i>Terminalia chebula</i> Retz.	Kadukai	Fruit	Muscular dislocation	KT	f
	<i>Terminalia chebula</i> Retz.	Kadukkai maram	Leaves	Cold, Cough, Fever, stomach-ache	PL	e
75	<i>Terminalia crenulata</i> Heyne ex Roth	Karimathi	Bark	Internal bleeding	KT	f
Compositae						
76	<i>Bidens pilosa</i> L., Katu	Katu kunni	Leaves	White patches on the legs	KU	i
Convolvulaceae						
77	<i>Arogyrea hirsute</i> Wight & Arn.	Meenidal	Leaves	Male Child	KO	a
78	<i>Ipomoea alba</i> L.	Velutha	Leaves	Skin diseases	KU	h
79	<i>Melothria maderaspatana</i> Cogn.	Solapushni kai	Stem	Prolonged cough	KU	i
80	<i>Trichosanthes cucumerina</i> L.	Peyppadal	Fruit	Headaches	IR	g
Dioscoreaceae						
81	<i>Dioscorea oppositifolia</i> L. var. tomentosa.	Nurulai/Valli kilangu	Rhizome	Stomacache	PL	e
Ebenaceae						
82	<i>Diospyros ferrea</i> (Wild.) Bahk. Var. buxifolia	Veeraii	Fruit	Bood circulation	IR	g
Ericaceae						
83	<i>Gaultheria fragrantissima</i> Wall.	Ameerpan	Leaves	Headache Relieve body sprains and pains	KO	a
Erthoroxylaceae						
84	<i>Erythroxylum monogynum</i> Roxb.	Jeevadalli maram	Stem Bark	Acute skin disease	KU	b
	<i>Erythroxylum monogynum</i>	Jeevathalimara	Bark	Scabies	IR, PA	c
Euphorbiaceae						
85	<i>Acalypha fruticosa</i> Forsskal.	Chinni chedi	Leaves	Dysentery, skin disease	PL	e
86	<i>Acalypha indica</i> L.	Kuppaimeni	leaves	Ear pain, snake bite and scabies	IR	g

Table 1: Continued...

87	<i>Acalypha paniculata</i> Miq.	Paruva thazhai	Leaves	Pimples, stomachache	PL	e
88	<i>Breynia rhamnoides</i> , Muell	Poolan	Root & Leaves	White patches on the skin all over the body	KU	h
89	<i>Euphorbia antiquorum</i> L.	Sathura kalli	Latex	Body pain	PL	e
90	<i>Euphorbia hirta</i> L.	Ammanpatcharisi	Leaves & Latex	Pimples	KU	h
91	<i>Euphorbia rothiana</i>	Spreng, Kopot	Latex	Sores, grow hairs, insect repellent	KU	i
92	<i>Excoecaria agallocha</i> L.	Thillai	Latex	Antiseptic	IR	g
93	<i>Excoecaria crenulata</i> L.	Vellai thillai	Stem	Skin disease	PL	e
94	<i>Jatropha curcas</i> L.	Kaatu amanku	Leaves	Headache	KT	f
95	<i>Jatropha tanjorensis</i> Ellis & Saroja	Katamanukku	Latex	Antiseptic	IR	g
96	<i>Mallotus philippensis</i>	Chaneri mara	Bark	Stomach pain and diarrhoea	IR, PA	c
97	<i>Phyllanthus amarus</i> Schum. & Thonn.	Kila nelli	Whole plant	Jaundice	KT	f
	<i>Phyllanthus amarus</i> Schum. & Thonn.	Kizhanelli	leaves	Jaundice	IR	g
98	<i>Phyllanthus emblica</i> L.	Nelli	Fruit	Stomachache	KT	f
99	<i>Ricinus communis</i>	Kottamuthu	Bark	Quick delivery, sprains, breathing problems	IR, PA	c
100	<i>Ricinus communis</i> Linn.	Amanaku	Leaves, Seeds	Headache	KO	a
101	<i>Securinega virosa</i> (Willd.) Baill.	Pula	Root	Joint pain	KT	f
102	<i>Euphorbia rothiana</i> Spreng.	Kapsi	Leaves	Sudden sickness and giddiness	KO	a
Fabaceae						
103	<i>Acacia nilotica</i> (L.) Willd. ex. Del.	Karuvellam	leaves	Dysentery, burns or scalds	IR	g
104	<i>Albizia lebbek</i> (L.) Benth.	Vagai	seeds	Lesions of lepers	IR	g
105	<i>Caesalpinia bonduc</i> (L.) Roxb.	Kazhchikai	seeds	Hydrocele	IR	g
106	<i>Canavalia lineata</i> (Thunb.) DC.	Kozhiavarai	seeds	Several Disorders, General health	IR	g

Table 1: Continued...

107	<i>Clitoria ternatea</i> L.	Sangu pushpam		Infection of eyes, headache, snake bites	IR	g
108	<i>Dalbergia sissooides</i>	Veetimara	Stem	Acute diarrhoea	IR, PA	c
109	<i>Flemingia strobilifera</i> (L.) R. Br. ex Ait.	Kaduthuvarai	Whole plant	Mental disorders	KT	f
110	<i>Macrotyloma uniflorum</i> (Lam.) verdc	Kollu	Seeds	Abortifacient	PA	b
111	<i>Millettia splendens</i>	Manalikodi	Stem	Burns and scalds , acute diarrhoea	IR, PA	c
112	<i>Mucuna pruriens</i> (L.) DC.	Poonikali	seeds	Several Disorders, General health	IR	g
113	<i>Pongamia pinnata</i> (L.) Pierre	Pongan	seeds	Rheumatic disease	IR	g
114	<i>Pterocarpus marsupium</i>	Pennae pattae	Bark	Abortifacient	IR, PA	c
115	<i>Shuteria vestita</i> W&A	Kadu belaga	Leaves	Boils appearing on the skin	KU	h
116	<i>Tephrosia purpurea</i> (L.) Pers.	Averi		Headaches	IR	g
Gentianaceae						
117	<i>Enicostema axillare</i> (Lam.) Raynal	Vellarugu	Root	Toothaches	IR	g
Hypoxidaceae						
118	<i>Curculigo orchoides</i> Gaertn.	Nelapanai	Rhizome	Snake bite	IR-S	d
Labiatae						
119	<i>Coleus malabaricus</i>	Periya tulasi	Leaves	Asthma	KU	i
120	<i>Plectranthus nilghericus</i> Benth.	Sone gida	Whole Plant	Minor wounds	KU	i
Lamaceae						
121	<i>Geniosporum tenuiflorum</i> (L.) Merr.	Nilathulasi	Whole plant	Catfish bites	IR	g
122	<i>Leucas aspera</i> (Willd.) Link	Thumbai	Root	Tooth brush, resistant to snake poison	IR	g
123	<i>Anisochilus carnosus</i> (L.f.) Wallich.	Saeththupun thazhai	Leaves	Skin disease	PL	e
124	<i>Anisomeles malabarica</i> (L.) R. Br. Ex. Sims	Paei miratti	Stem	Wound	PL	e
125	<i>Coleus parviflorus</i> Benth	Nila	Tuber	Itching, boils on the skin	KU	h

Table 1: Continued...

126	<i>Leucas biflora</i> (Vahl.)R.Br.	Kaduthumbae	Whole plant	Skin irritations	KU	h
127	<i>Leucas indica</i> (L.) R. Br. ex Vatke	Mosappullu	Leaves	Cough	KT	f
128	<i>Ocimum basilicum</i> Var purpurasens	Katu thulasi	Whole plant	Skin inflammations after the insect bites	KU	h
129	<i>Plectranthus coleoides</i> Benth.	Mudupattan or Omavalli chedi	Leaves	Cold, delivery pain, hair growth, wounds	PL	e
130	<i>Plectranthus malabaricus</i> (Benth.) R.H. Willemse	Ellamabai	Leaves	Heart attack	IR-S	d
131	<i>Prunella vulgaris</i> Linn.	Kadthur	Root	Refrigerant Haematonic	KO	a
132	<i>Aloe vera</i> (L.) Burm.f	Sotru Kattrazhai	Leaves	Hair and skin Diseases	KU	h
133	<i>Asparagus racemosus</i> Willd.	Thanneer vittan kilangu	Leaves	Heel cracks	PL	e
Lobeliaceae						
134	<i>Lobelia heyneana</i> Roem. & Schult.	Upperi chedi	Leaves, Flowers	Skin disease	PL	e
135	<i>Lobelia leschenaultiana</i> (Presl) Skottsb.	Bombari thalai.	Whole Plant	Sickness in cattle	KO	a
Loganiaceae						
136	<i>Strychnos nux-vomica</i>	Yetti	Bark	Acute stomach pain	IR, PA	c
Lythraceae						
137	<i>Lagestroemia microcarpa</i> Wight	Tindiyam	Bark	Burns	KT	f
Malvaceae						
138	<i>Hibiscus rosa sinensis</i> L.	Chembarathi	Flowers	Strengthening hair	KU	h
139	<i>Malvatum coromandelianum</i> (L) Garke	Kalakenikai	Root	Stomach pain	IR-S	d
140	<i>Sida acuta</i> Burm f.	Pilla valatthi chedi.	Leaves	Dandruffs , strengthening hair	PL	e
141	<i>Sida rhombifolia</i> L.	Kal gadale	Leaves	Wounds	KU	i
	<i>Sida rhombifolia</i> L.	Chitra mutti	Root	Rheumatic pain	KT	f
142	<i>Sida cordifolia</i> L.	Arathae	Leaves	Snakebite	PA	b
Meliaceae						
143	<i>Azadirachta indica</i>	Veppamaram	Stem	Toothache, post-natal complications	IR, PA	c

Table 1: Continued...

144	<i>Azadirachta indica</i> A. Juss.	Veppam	leaves	Stomach worms	IR	g
145	<i>Cipadessa baccifera</i> (Roth.) Miq.	Pulipancheddi	Leaves	Unconsciousness and anaemia	KT	f
	<i>Cipadessa baccifera</i> (Roth.) Miq.	Pulippan chedi	Leaves	Diarrhoea	PL	e
	<i>Cipadessa baccifera</i> (Roth.) Miq.	Marundha soppu	Leaves	Rheumatism	IR-S	d
Menispermaceae						
146	<i>Cissampelos pareira</i> L. var. <i>hirsuta</i> (Ham. ex DC.) Forman	Urikkakodi	Tuber	Snakebite	KT	f
147	<i>Cissampelos pureira</i> , L.	Koodibatale	Leaves	Headache, fever, burning sensation in chest	KU	i
148	<i>Cyclea peltata</i> (Lam.) Hook. f. & Thomson	Para	Tuber	Body pain	KT	f
	<i>Cyclea peltata</i> (Lam.) Hook.f.	Sethari Kodi	Leaves	Cough, cold and body pain	IR-S	d
149	<i>Tinospora cordifolia</i> (Wild) Miers ex Hook.F. & Thoms	Amrithavalli	Leaves	White rashes appearing on the body	KU	h
Mimosaceae						
150	<i>Acacia caesia</i> (L.) Willd.	Nanjupattai	Bark	Wound	PL	e
	<i>Acacia caesia</i> (L.) Willd.	Kari Indu	Stem bark	Body pain	KT	f
151	<i>Acacia leucophloea</i> (Roxb.) Willd.	Sarayapattai maram	Bark	Cuts	PL	e
152	<i>Mimosa pudica</i> L. (Mimosaceae)	Thotalvadi	Whole plant	Body pain	KT	f
Moraceae						
153	<i>Ficus infectoria</i> Roxb	Selakai	Fruit	Food	KU	i
154	<i>Ficus racemosa</i> L	Athikai	Fruit	Eye sight	KU	i
Moringaceae						
155	<i>Moringa concanensis</i> Nimmo	Kattu murukka	Bark	Abortifacient	IR, PA	c
156	<i>Moringa oleifera</i>	Murunga	Bark	Dog or scorpion bite	IR, PA	c
Musaceae						
157	<i>Musa paradisiaca</i>	Vazhai	Stem	Acute diarrhoea	IR, PA	c

Table 1: Continued...

Myrataceae						
158	<i>Eucalyptus polybractea</i> R. T Baker	Karpura mara	Leaves & bark	Round patches between fingers	KU	h
159	<i>Psidium guajava</i> L.	Koyyapazham	Fruit	Gastric troubles and ulcers in stomach	PA	b
	<i>Psidium guajava</i> L.	Koyyapazham	Fruit & Leaves	Anti-dysenteric and Antidiarrhoeal	KU	i
160	<i>Syzygium cumini</i> (L.)	Skeels Naval Pazham,	Stem Bark, Fruit & Seeds	Sore throat, dysentery, ulcers, purifying blood, antidiarrhoeal and anti diabetic	KU	i
	<i>Syzygium cumini</i> (L.) Skeels	Naval	bark	Diarrhoea	IR	g
Nyctaginaceae						
161	<i>Mirabilis jalapa</i> L.	Thottanembi	Root , Leaves	Cuts and wounds	KO	b
Orchidaceae						
162	<i>Cymbidium aloifolium</i> (L.) Sw.	Ottai	Root	Ear pain	IR	g
163	<i>Malaxis densiflora</i> (A. Rich.) Kuntze	Kuntze, Nelnethch	Leaves	Wounds	KU	i
Oxalidaceae						
164	<i>Oxalis corniculata</i> L.	Pulichen segae	Whole Plant	Febrifuge	PA	b
	<i>Oxalis corniculata</i> L.	Puliyankeraai	Leaves	Vomiting and headache	IR-S	d
	<i>Oxalis corniculata</i> Linn.	Pulch	Leaves	Anti-emetic Restorative tonic after child birth	KO	a
Pandanaceae						
165	<i>Pandanus odoratissimus</i>	Kaithae	Stem	Fracture	IR, PA	c
166	<i>Passiflora calcarata</i> Mast	Potul	Leaves	Skin diseases	KU	h
167	<i>Passiflora foetida</i> L.	Narati chedi	Whole Plant	Arthritic problems	KU	b
Periplocaceae						
168	<i>Hemidesmus indicus</i> (L.) R. Br.	Nannari	Root	Mouth ulcers	KT	f
Piperaceae						
169	<i>Piper betle</i> L.	Thabulam	Whole plant	Cuts and wounds	KT	f

Table 1: Continued...

170	<i>Piper brachystachyum</i> Wall.,	Kadu kurumulaku	Fruit	Cough	KU	i
171	<i>Piper nigrum</i> L.	Milagu	Seeds	Throat infection	PL	e
Plantaginaceae						
172	<i>Plantago erosa</i> Wall.	Kalthal	Leaves	Wounds as a antiseptic	KO	a
173	<i>Plantago lanceolata</i> L.	Neela kare	Leaves	Boils on the legs	KU	h
Plumbaginaceae						
174	<i>Plumbago zeylanica</i> L.	Chitthira moolam	Root	Stomachache	PL	e
	<i>Plumbago zeylanica</i> L.	Cithiramalliver.	Root	Insect bite	IR-S	d
Poaceae						
175	<i>Cymbopogon citratus</i> L.	Karppura pul	Root	Pimples	KU	h
176	<i>Cynodon dactylon</i> (L.) Pers.	Arugampul	Branches	Body coolant	IR	g
Polygonaceae						
177	<i>Rumex nepalensis</i> Spreng.	Gundott or Sukkutu	Root	Refrigerant and laxative	KO	a
	<i>Rumex nepalensis</i> spreng.	Keerai				
		Kekal Ott, Gund Ott	Root	Jaundice	KO	b
Proaceae						
178	<i>Cynodon dactylon</i> (Linn.) Pers.	Nagiriki	Leaves	Relief from sudden sickness	KO	a
Ranunculaceae						
179	<i>Clematis gauriana</i> Roxb.	Meenae	Leaves & stem	Wounds & skin Diseases	KU	h
	<i>Clematis gouriana</i> Roxb. Ex. DC.	Attumeesai chedi	Leaves	Skin disease	PL	e
Rhamnaceae						
180	<i>Ziziphus mauritiana</i>	Yelluchi maram	Bark	Gastric disturbance	IR, PA	c
	<i>Ziziphus mauritiana</i> Lam.	Elanthai	Bark	Old wounds	IR	g
	<i>Ziziphus mauritiana</i> Lam.	Ilantha	Whole Plant	Mouth freshener	KT	f
Rubiaceae						
181	<i>Catunaregam spinosa</i> (Thunb.) Tirvengadum	Madukarei	Root	Ulcers	KT	f
182	<i>Rubia cordifolia</i> L.	Sappli Koth	Stem	Restorative, jaundice	KO	b

Table 1: Continued...

	<i>Rubia cordifolia</i> L.	Kalutharupan chedi	Leaves	Heel cracks	PL	e
	<i>Rubia cordifolia</i> L.	Periya nangai.	Leaves	Cough, cold and nervous disorders	IR-S	d
	<i>Rubia cordifolia</i> Linn.	Maral	Leaves	Injuries caused by fire	KU	i
	<i>Rubia cordifolia</i> L.	Muthang	Root	Dysmenorrhoea	KT	b
Rutaceae						
183	<i>Citrus aurantium</i> L.	Eravae kai	Fruit	Digestion, Hemorrhoids	KU	i
184	<i>Clausena dentata</i> (Willd.) Roem.	Anai thazhai	Leaves	Wound	PL	e
185	<i>Glycosmis mauritiana</i> (Lam.) Yaich.	Panasedi	Leaves	Headache	IR-S	d
186	<i>Glycosmis pentaphylla</i> (retz.) DC.	Eruputtal	Whole Plant	Stomach ache and abdominal discomfort	KT	b
187	<i>Murraya koenigii</i> L.	Karivepilla	Leaves	Skin inflammations	KU	h
188	<i>Murraya paniculata</i>	Chedichi	Bark	Toothache	IR, PA	c
189	<i>Naringi crenulata</i> (Roxb) Nicolson	Naivalampattai	Bark	General health	IR-S	d
190	<i>Ruta chalepensi</i> L.	Aruvatha Geeda	Leaves	Infant convulsions	KO	b
191	<i>Ruta graveolens</i> L.	Aruvadam	Leaves	Skin diseases	KU	h
	<i>Ruta graveolens</i> L.	Arubathansedi	Leaves	Diarrhea, stomach pain and vomiting	IR-S	d
192	<i>Toddalia asiatica</i> (L.) Lam.	Surai	leaves, Fuit	Fever, headache	IR	g
	<i>Toddalia asiatica</i> (L.) Lam.	Kindu mullu	Leaves, stem, Root bark	Stomachache, toothache	PL	e
	<i>Toddalia asiatica</i> (Linn). Lam.	Vaseri	Leaves , Seeds	Vermifuge	KO	a
Salvadoraceae						
193	<i>Azima tetracantha</i> Lam.	Sankan	leaves	Fever	IR	g
194	<i>Salvadora persica</i> L.	Vagai	Fruit, Root	Rheumatic pains, tooth brush	IR	g
Santalaceae						
195	<i>Santalum album</i>	Santhana mara	Heartwood	Refrigerant, skin diseases	IR, PA	c

Table 1: Continued...

	<i>Santalum album</i> L.	Sanddanamara	Seed	Skin troubles, refrigerant	KU	i
196	<i>Thesium wightianum</i> Wall. ex Wight	Anaikchi	Whole Plant	Cheek to prevent bulging	IR-S	d
Sapindaceae						
197	<i>Cardiospermum halicacabum</i> L.	Mudakkathan	whole plant	Rheumatoid arthritis	IR	g
	<i>Cardiospermum halicacabum</i> L.	Poovanthi	Nuts	Body wash	IR	g
198	<i>Dodonaea angustifolia</i> L.f.	Marundha soppu	Leaves	Rheumatism	IR-S	d
199	<i>Dodonaea viscosa</i> (Linn.) Jacq.	Vilari thalai	Leaves	Wounds and injuries, joint sprains and bone fracture	KO	a
200	<i>Dodonea viscosa</i> Linn.	Manantha	Leaves	Fracture	KU	i
201	<i>Schleichera oleosa</i>	Jagada mara	Bark	Abortifacient	IR, PA	c
Sapotaceae						
202	<i>Manilkara hexandra</i> (Roxb.) Dubard	Pala maram	Latex	Toothaches	IR	g
203	<i>Tinospora cordifolia</i> (Willd.) Miers	Seenthil	stem	Many Disorders	IR	g
Simaroubaceae						
204	<i>Ailanthus excelsa</i> Roxb.	Pekalathi	Bark, leaves	After child birth	IR	g
Solanaceae						
205	<i>Datura stramonium</i> L.	Umbathi	Leaves	Inflamed wound and sores	KO	b
	<i>Datura stramonium</i> L.	Yemmuth	Leaves & Fruit	Piles	KU	i
206	<i>Physalis peruviana</i> L.	Urechithuvar	Leaves	Wound	KU	i
207	<i>Solanum anguivi</i> Lam.	Kandan kathiri	Fruit, leaves	Colds, coughs and fever, intestinal worms	IR	g
208	<i>Solanum denticulatum</i>	Periya midinje	Whole Plant	Migraine	KU	i
209	<i>Solanum erianthum</i> D.Don	Malai sundai	Fruit	Toothache	PL	e
210	<i>Solanum indicum</i> Linn.	Sunda maram	Root & Leaves	Toothache and snakebite	KU	i
211	<i>Solanum nigrum</i> L.	Mana thakkali	Leaves	Ulcer, wound	PL	e
	<i>Solanum nigrum</i> Linn.	Ikki sop	Leaves	Stomach disorders Skin rashes	KO	a
212	<i>Solanum sisymbirifolium</i> Limk.	Vadadana	Seeds	Vermifuge	KO	a
213	<i>Solanum surattrense</i> Burm. F	Kandankathiri	Fruit	Toothache	PL	e

Table 1: Continued...

214	<i>Solanum trilobatum</i> L. Tamaricaceae	Thoodhuvalai	Leaves	Asthma, Cold	PL	e
215	<i>Tamarix indica</i> willd Thunbergiaceae	Kattuchaukku	leaves	Laxative	IR	g
216	<i>Thunbergia fragrans</i> Roxb. Tiliaceae	Kakka Valli	Root	Snake-bite	KT	b
217	<i>Grewia aspera</i> Roxb	Dadchi maram	Bark	Diarrhoea	KU	i
218	<i>Grewia tiliifolia</i> Vahl. Ulmaceae	Unu	Root bark	Swellings	KT	f
219	<i>Holoptelea integrifolia</i> Umbelliferae	Vellaya	Bark	Swellings	IR, PA	c
220	<i>Centella asiatica</i> (L.) Urban. Verbenaceae	Vallarai	Leaves	Jaundice	PL	e
221	<i>Gmelina arborea</i> Roxb	Perungilai/ Kumilamaram	Root bark	Piles	PL	e
222	<i>Gmelina asiatica</i> L.	Kumalai Nochi	Fruit	Bathing	IR	g
223	<i>Lantana camara</i> L.	Unni chedi	Whole plant	Cuts and wounds	KT	f
	<i>Lantana camara</i> L.	Thusik	Leaves	Gum bleeding and tooth-ache	KO	b
	<i>Lantana camera</i> L.	Parale gida	Flowers	Skin inflammations	KU	h
224	<i>Tectona grandis</i>	Thekku	Bark	Constipation	IR, PA	c
	<i>Tectona grandis</i> F.	Thekku	Bark	Ease child birth and labour pain	KU	b
	<i>Tectona grandis</i> L.	Thekku	Bark	Ease child birth and labour pain	PA	b
	<i>Tectona grandis</i> L.f.	Thekku	Stem	Stomach ache and dysentery	KT	f
225	<i>Vitex negundo</i> L.	Nochhi	Leaves	Rejunvating skin	KU	h
	<i>Vitex negundo</i> L.	Kumalai Nochi	leaves	Repel mosquitoes, body pain	IR	g
	<i>Vitex negundo</i> L.	Notchi	Fruit	Cold, Cough, Fever, headache	PL	e

Table 1: Continued...

Zingiberaceae

226	<i>Alpinia calcarata</i> Rosc.	Arathi poo	Rhizome	Immunity	PL	e
227	<i>Costus speciosus</i> (J. Koen.) Smith.	Koshtam	Leaves	Diabetes	PL	e
228	<i>Curcuma longa</i> L.	Manjal	Rhizome	Itching, glowing skin	KU	h
	<i>Curcuma longa</i> L.	Manjal	Rhizome	Scorpion bite	KT	f
229	<i>Elatteria cardamomum</i> (L.) Maton.	Yelakkai	Fruit	Stomachache	PL	e

KU = Kurumba, IR = Irula, KT = Kota, TH = Thoda, PA = Paniya, PL = Paliyar, KT = Kattunayakas. a, (Rajan and Sethuraman, 1991); b, (Rajan et al., 1997); c, (Rajan et al., 2001); d, (Murugesan et al., 2005); e, (Ignacimuthu et al., 2006); f, (Udayan et al., 2007); g, (Ragupathy and Newmaster, 2009); h, (Deepak et al., 2014a); I, (Deepak et al., 2014b)

Thus, the medicinal uses of the tribal traditional medicines depict their medical history through the ages. They have tried and found cure to their major health problems such as wounds and skin problems. This data also throws light on a cultural aspect of their life style, mainly that they held their women in high esteem for they have painstakingly developed remedies form medicinal plants to alleviate labour pains and post-partum medical care.

6. Towards sustainable local production of traditional medicines

Sustainable development denotes a development that meets the needs of the present without compromising the ability of future generations to meet their own needs. It encompasses two key concepts, the concept of needs, in particular meeting the essential needs of the poor and the idea of limitations that is the environment's ability to meet these needs. Thus sustainable development seeks to relieve poverty, create equitable standards of living, satisfy the basic needs of all peoples, and establish sustainable political practices, while ensuring that there are no irreversible damages to natural resources and nature.

The tropical countries are gifted with vast resources of medicinal plants and the recent global renaissance in traditional medicines has created a large market for herbal products that can be exploited by these countries they meet up to quality and safety specifications. Population explosion, incidence of side effects of synthetic medicines and our inability to provide modern medicines to a vast section of the population living in rural and remote areas of the country due to non-availability, in accessibility and unaffordability have been the prime reasons for the growing popularity of alternative medicines amongst rural and remote population, and neo-rich people in the developed countries. Access to quality health care is an enormous public health global issue at the scientific, clinical, economic, political and policy levels. It is one aspect of the "great divide" that exists between and within every country in the world; it is the difference in access to health care between the rich and the poor. The very basis for promoting the local production of herbal remedies is to provide cost effective medicines to populations who cannot afford costly medicines. The World Health Organization (WHO) has been repeatedly stressing that the goal of 'health for all' cannot be accomplished without herbal medicines.

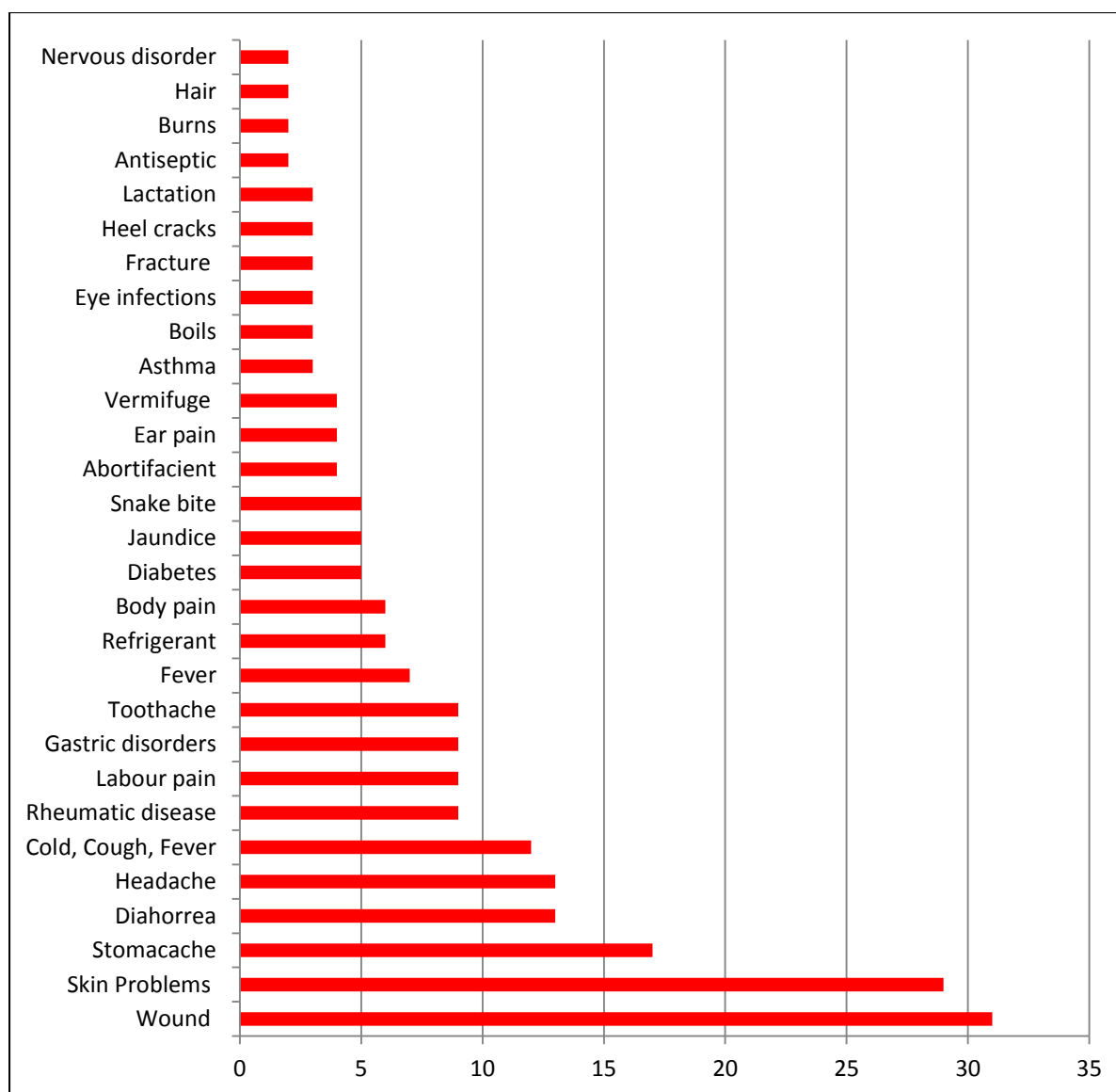


Figure 3: Traditional Medicine of the Tribes of Tamil Nadu in the treatment of common ailments.

7. Strategies for promoting sustainable production of traditional medicines

The first step in promoting sustainable production of traditional medicine is developing a standardized mode of production which will meet the standards of quality, efficacy and safety as defined in the WHO guidelines. Thus bringing these plants into large scale cultivation ensures that endangered plant are protected by cultivation and by involving the indigenous people over-exploitation will be avoided while adding to income for the indigenous tribes. Majority of the plants are still gath-

ered and collected from the wild and relatively few are cultivated in farmlands. World Wildlife fund report (2004) reported that 20% of the medical plants worldwide are in the treat of disappearing (Pan *et al.*, 2013). A paradigm shift from wild collection to structured cultivation of medicinally important plants will further ensure the purity, authenticity and sustainable supply of raw drugs. Suitable infrastructure like production equipment, potable water, storage facilities and post-harvest quality monitoring and marketing are very critical. Trained man power with an appropriate background in pharmaceu-

tical sciences in bioprocess technology is an integral part of the quality of the finished product (Cordell and Colvard, 2012). The concept of sustainability directs us to shift from non-renewable chemical drug discovery programs to natural renewable sources (Cordell, 2011). Sustainable production of traditional medicine will require an integrated approach encompassing the different disciplines of science like ethnobotany, chemistry, biomedicine, mathematics and physics. Traditional medicine has still not come to the forefront because of the following challenges faced by the pharmaceutical companies in providing the capital investment in developing and marketing them namely, collection of plants is a time consuming process and requiring extensive negotiations related to access, insufficient documentation and lack of ethnomedical knowledge, lack of institutional and financial support, limited availability of trained manpower, low yield, long discovery process and expensive synthesis, lack of scientific validation of the quality, safety and efficacy of traditional formulations, lack of appropriate technology for post-harvest and pre-processing purposes, low market value for traditional medicinal plants, lack of methodologies for the preservation of medicinal extracts for extended shelf life.

8. Biotechnology: challenges and prospects for the sustainable use of traditional medicine

Biotechnology with its robust tools and state of art technology will play a crucial role in the sustainable use of traditional medicinal drugs in future. Although the use of transgenic plants is a debatable for the preservation of biodiversity, genetic engineering will play an important role in saving medicinal plants, which are rare or endangered (Cordell, 2011). Ethnomedical information about biological evaluation of plant extracts and their constituents, the chemistry of natural sources, and the clinical evaluation of plant extracts are still not

accessible globally. Biotechnological aspect of herbal remedies involves extraction of plant's active ingredients and formulation into final product. First, it must be established unequivocally that the source of the plant material is authentic. A plant extract usually contains hundreds of active ingredients and in some cases the bioactive compound is usually not known. Bioactivity guided processes are then used to separate the active compound. This is further tested on in-vitro or in-vivo models for their functional efficacy. There are many disease conditions for which such biological models are not easily available, or if even available, would be beyond the means of many researchers. Therefore a more practical and cost effective way is to use total extracts of traditional medicinal plants for which abundant ethnomedical evidence exists. The mode of formulation can then be based on the traditional methods used to prepare that particular remedy, taking steps to establish safety through in vivo and in vitro studies followed by appropriate pilot clinical trials for cytotoxic, mutagenic and therapeutic perspectives. The formulation should be free of potentially toxic insecticides, pesticides and heavy metals. The formulation must be evaluated for microbial contamination both fungal and bacterial, and radiation contamination during the stages of processing of the material (Tan *et al.*, 2004). Quality control of the product is then done by conducting accelerated stability tests as well as on-shelf stability tests.

The pharmacological approach in developing a drug includes bringing as much chemical diversity as possible to the biological screening interface but with no consideration given to the origin of the plant derived materials, chemo-diversity, functional diversity of the constituents, ethno-medical association of the plant or known or novel active constituent of the plant extracts (Tan *et al.*, 2004). The other challenge is that the active constituent is often extracted and analyzed only at a single point in time, ignoring daily metabolic

flux, seasonal variation in enzyme activities, and the biosynthetic genes which are present, but not fully functional. Methodologies that are able to characterise the majority of the constituents without individual isolation of active constituents need to be developed to help validation and standardization. India has a vast and diverse wealth of traditional medicinal knowledge that can be used for bioprospecting to benefit both the country and the indigenous people.

9. Bioprospecting of traditional medicine to combat disease

The search for new drugs is the vast opportunity in the ambit of Biotechnology given the vast majority of diseases encountered today and improved health care services.

There are three main types of search strategies: biorational, chemorational and random approaches. An anti-HIV bioactive compound Conocurvone was discovered as a result of random approach of screening strategies. Drugs discovered using bio-rational approaches were artemisinin, morphine, quinine, and ephedrine. Bio-rational approach is mostly guided by the ethnomedical information generated from the traditional medicines and the most effective approach to date. These medicinal plants contain reservoir of etho-medical and ethno-botanical traditional knowledge, which is an important guide to discovery of many new drug lead molecules (Table 2). As there are many existing and emerging diseases that cannot be treated by the current plethora of drugs and the additional burden of increasing

Table 2: Bioactive compounds from medicinal plants and their clinical uses

SN	Bioactive compound	Species	Clinical Uses
1	Mevastatin & lovastatin	<i>Penicillin spp.</i>	Cholesterol lowering
2	Ivermectins	<i>Streptomyces spp.</i>	Anthelmintic and antiparasitic
3	Reserpine	<i>Rauwolfia serpentina</i>	Antihypertensive
4	Ephedrine	<i>Ephedra sinca</i>	Antiasthma
5	Atropine	<i>Belladonna</i>	Anticholinergic
6	Teprotide	<i>Bothrops jaracaca</i>	Cardiovascular diseases
7	Vincristine and vinblastine	<i>Catharanthus roseus</i>	Anti-cancer drug
8	Paclitaxel	<i>Taxus brevifolia</i>	Anti-cancer drug
9	Camptothecin	<i>Camptotheca acuminata</i>	Anti-cancer drug
10	Podophylotoxin	<i>Podophyllum peltatum</i>	Skin Cancer
11	Bryostatin-1	<i>Bugula neritina</i>	Ovarian carcinoma and non-Hodgkin's lymphoma
12	Cyclosporins and rapamycin	<i>Penicillium notatum</i>	Antimicrobial and anti-plasmodial

drug resistant pathogens, the need for the hour is the development of new arsenal of drugs to combat them based on the traditional knowledge.

10. Biotechnological tools to be exploited for sustainable production of traditional medicines

Biotechnology produces a vast array of tools for the successful discovery and validation of traditional medical drugs. These tools have been developed in the 80's and 90's and today there is vast improvement in their technological aspects after subsequent modification of the initial technology. Each technique has its own pros and cons and care needs to exercise in the employment of a suitable technology to provide an appropriate traditional medicine based drug. Techniques like in vitro regeneration through micropropagation, callus mediated organogenesis, somatic embryogenesis, cryopreservation, production of secondary metabolites and genetic transformation holds a tremendous potential for the production of high quality plant based medicines mainly because of the multiplication rate, pathogen free material, plant preservation and regeneration success to yield bioactive compound that ensures reduced costs compared to the natural synthesis by the plants.

11. Intellectual property rights (IPR) for sustainable use of traditional medicine

It is very vital to consider intellectual property rights of the local people when the ethnomedical documentation is done for traditional medical knowledge. The economic implication of the eventual commercial production of standardized traditional medicinal drug should include the welfare of the people from whom the traditional knowledge was documented. Biopiracy becomes an ever looming issue when large pharmaceutical companies confiscate medicinal plants to make new

drugs, the costs escalate and these drugs will not be available to the local people because of the costs which is also a critical an ethical issue (Pan et al., 2013).

11.1 The Kani model of benefit sharing of traditional medicine

The Kanis inhabit the forests of the Thiruvananthapuram district of Kerala and Thirunelveli district of Tamil Nadu in southwestern India. In 1987, scientists from Tropical Botanic Garden and Research Institute (TBGRI) while collecting ethnomedical data from Kani tribal people discovered that the tender fruits of Arogyappacha (*Trichopus zeylanicus* subsp. *travancoricus*) have anti-ageing, anti-depressant and anti-fatigue property. This paved a way for the scientists at TBGRI to develop a scientifically validated and standardised herbal drug called Jeevani, a formulation consisting of four ingredients and Arogyappacha was one of the constituents. Jeevani has been found to be therapeutically effective having anti-fatigue and immuno-enhancing properties and it has also shown good hepato-protective and anti-stress properties. Subsequently, TBGRI decided to share 50% of the licence fee and royalty with the Kani people to encourage an equitable sharing of the benefits arising from the utilisation of such knowledge, innovations and practices as stated in the mandate of Article 8(j) of the Convention on Biological Diversity (CBD). This is considered to be one of the first models for benefit sharing in the world, which is popularly known as the TBGRI Model for Benefit Sharing.

12. Conclusion

Sustainable local production of traditional medicines requires an enabling environment and effective partnerships between traditional health practitioners, researchers, public and the private sector. There is a strong need for indexing eco and ethno information of medicinal plants, sustainable cultivation of the traditional medi-

cal plants, protecting the intellectual property rights of the indigenous people and using the various tools of Biotechnology in formulating a superior product that is cost effect to the very same indigenous people. Recent innovations in Biotechnology have impacted the use of molecular tools for sustainable use of a renewable natural resource for the betterment of India and its indigenous communities. Modern tools of Genomics can be applied to traditional medicine without the need for transgenics. Protection of intellectual property rights of the traditional medicinal knowledge of indigenous people in the form of benefit sharing and bioprospecting the vast biodiversity available in the country is the need of the hour to march India in the field of bio pharmaceuticals as a global leader. It is now in the hands of young researchers to work under well placed regulatory framework in converting traditional medicinal knowledge into a safe and novel bio drug for the treatment of existing and emerging disorders.

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Vermitechnology – An Eco-Biological Tool for Sustainable Environment

Mahaly Moorthi¹, Koilpathu Senthil Kumar Abbiramy^{1*}, Arumugam Senthil Kumar² and Karupannan Nagarajan³

¹PG and Research Department of Zoology & Wildlife Biology, A.V.C. College (Autonomous), Mannampandal - 609305, Mayiladuthurai, Nagapattinam District, Tamilnadu, India; ²PG and Research Department of Zoology, Chikkaiah Naicker College, Erode – 638 004, Tamilnadu, India; ³PG and Research Department of Zoology, Sri Vasavi College, Erode – 638 316, Tamilnadu, India; *Correspondance: moorthideksha@gmail.com / ksabiramy@gmail.com; Tel; +91-8526385977

Abstract: The word vermi, typically indicates earthworms. Vermitechnology is a simple process, which uses earthworms to produce earthworms, good quality compost (vermicompost) through organic waste recycling and other products involving earthworms. This technology is inevitable in managing biodegradable wastes, biomass or organic material that can be degraded or composted thus contributing to the environment indirectly. Solid waste management through Vermitechnology contributes more for the sustainability of the environment. The major components of Vermitechnology can be considered as Vermiculture (mass production of earthworms), Vermicomposting (production of vermicompost) and Vermiwash (the extract of vermicompost). In 1996, 'Vermitech', the vermicomposting technique was developed by Mr. A. Thimmaiah at the Indian Agricultural Research Institute (IARI), New Delhi, India. As IARI is also known as 'Pusa Institute', this innovative technology was dedicated to the institute and named 'Pusa Vermitech'. 'Pusa Vermitech' was developed to provide a simple solution to poor farmers. This method has now become popular in Bhutan, Costa Rica, India, Italy, Nepal and Sri Lanka. It appears that Vermitechnology is going to play an important role for the sustainability of agriculture and environment. This chapter is highlighting the Vermitechnology, as an eco-biological tool for the sustainable environment.

Keywords: Solid waste management; vermicomposting techniques; vermiculture; vermitechnology; vermiwash

1. Introduction

The advent of organic farming has made farmers innovative and nature friendly. Vermitechnology, an effective replacement for chemical input is the most sought after due to its cost-effectiveness and quality of enriching the soil. Vermicompost is becoming the principal manure for crops in the field of organic farming. The market crisis for agricultural products has also contributed to the popularity of vermicomposting. The

manure comes in handy especially for small-holders who do not have the money to buy expensive, chemical fertilizers. The manure is used rampantly for all sorts of crops. The biggest beneficiaries are women who have formed themselves into NGO-trained Self-Help Groups (SHGs). These women can easily prepare the Vermitech products in their backyard and sell the excess one left after their own use to neighbouring estates or farmers. The NGO-run institutes like these are assisted by the local bodies like grama pancha-

yats, zilla panchayats, which organize seminars and teach farmers the methods of Vermitechnology. Government agricultural departments also buy earthworms in bulk from these SHGs to conduct their own projects on vermiculture. In fact, the Supreme Court's ruling criteria is that to decide on metro status would be the particular district's level of participation in organic farming.

But the low sense of awareness still remains a problem even within the municipalities that have undergone seminars on Vermitechnology. Neither the Boards nor the local bodies have ideas on the perspectives of Vermitechnology. Technical expertise alone does not make organic farming. Unless there is a uniformity of procedure, the advantages of organic farming – low cost, more soil fertility and eco-friendliness - will not come through.

After all, by preparing Vermitech products, the farmer is making the soil healthy. In turn, he's supplying healthy crops into the market. The content of organic carbon, the index for the presence of humus in the soil, is in high Ranges. So the farmers must contribute considerably more towards ecology and food production. He deserves all the support he can get. With the right financial support from the Government and a more organized network of cultural units, Vermitech products, as a form of enrichment can generate a steady source of income for the impoverished folk of agricultural areas.

There are numerous sources of waste produced in India where degradable organic matter is either partially or fully generated. Solid waste consists of the discarded portion of the households, dead animals, trade, commercial, agricultural and industrial waste and other large waste like debris from construction site, furniture etc. Solid wastes are generally categorized as domestic, industrial and hazardous or biomedical waste. Studies were made on some solid wastes like sewage sledges and solids from waste water (Mitchell *et al.*, 1980); wastes from pro-

cessed potato; wastes from supermarkets and restaurants; wastes from poultry, pigs, cattles, sheeps, goats, horses (Edwards and Bate, 1992) as well as horticultural residues from dead plants and spent wastes from mushroom industry (Edwards, 1988).

The degradable organic matter from these wastes when dumped in open undergoes either aerobic or anaerobic degradation. These un-engineered dumpsites permit fine organic matter to become mixed with percolating water to form leachate. The potential for this leachate to pollute adjoining water and soil is high. India where a lot of solid organic waste is available in different sectors with no dearth of manpower, the environmentally acceptable Vermitechnology using earthworms can very well be adopted for converting waste into wealth. Considerable work has been carried out on vermicomposting of various organic materials and it has been established that epigeic forms of earthworms can hasten the composting process to a significant extent, with production of a better quality of composts as compared with those prepared through traditional methods. The viability of using earthworms as a treatment or management technique for numerous organic waste streams has been investigated by a number of workers (Logsdon, 1994; Madan, 1988; Singh, 2002). Similarly a number of industrial wastes have been vermicomposted and turned into nutrient rich manure (Sundaravadivel, 1995). Hand *et al.* (1988) defined vermicomposting as a low cost technology system for the processing or treatment of organic wastes.

A growing awareness of some of the adverse economic and environmental impacts of agrochemicals in crop production has stimulated greater interest in the utilization of organic amendments such as compost or vermicompost for crop production (Follet, 1981). Therefore, the sustainability has to be restored by some means of regular food security. Utilization of earthworms may be an answer as

an ecologically sound, economically viable and socially acceptable technology. The present chapter reviews on various aspects involved in Vermitechnology and thus managing the organic waste leading to a sustainable environment.

2. Vermiculture

The process of culturing of earthworms using scientific methods is known as Vermiculture. Earthworms are known as biological indicators of soil health. In soil earthworms' activity and their casting support a lot amount of microbial populations. Microbial populations such as bacteria, fungi, Actinomycetes and protozoans grow well, also insects like spiders, millipedes, and other nematodes that are essential for sustaining the soil fertility grow well. Presence of soil biome enriches the soil fertility. Thus earthworms which form the base for the survival of other organisms can be cultured artificially and used for many purposes. The ultimate goal of this technology is the betterment of soil fertility and health of human beings.

In recent years considerable attention has been focused upon the potential role of intensive earthworm culture, or vermiculture. It is now accepted that the economic value of vermiculture lies in (i) reduction of noxious qualities associated with organic wastes, e.g. elimination of smell; (ii) generation of a useful compost; and (iii) production of earthworm biomass. Various Vermiculture systems, which have been designed primarily for biological waste control, are producing earthworms in large quantities. This chapter covers the production of the earthworms which can be used as a source of food, primary proteins, and as drugs.

Now-a-days, people are very much eager in culturing earthworms as a part time business, as a source of income. The culturing of worms becomes a promising business because of its need in enormous amount in organic farming, in big municipalities for the treatment of sol-

id wastes (John Paul *et al.*, 2011), as a source of live feed in poultry and aquaculture industries. Worms have a number of other possible uses on farms, including value as a high quality animal feed. The earthworms are also used as bait in freshwater sport fishing. They are also sold to small scale business people who maintain garden at home and to nurseries that do organic gardening and composting and sell saplings. Also there are demands for the pure form of vermicastings in various places.

2.1. Methods for Vermiculture

Vermiculture is the culture of earthworms. The goal is to continually increase the number of worms in order to obtain a sustainable harvest. The worms are either used to expand a vermicomposting operation or sold to customers who use them for the same or other purposes. If the goal is to produce vermicompost then we want to have maximum worm population density all of the time. If the goal is to produce worms then we keep the population density low enough that reproductive rates are optimized. Vermiculture as a business must be started in a small scale units. After learning the technique of culturing, one can start his business at large scale. Culturing of earthworms needs minimum requirements and care on a regular schedule. Culturing can be done by two methods.

- i. Container method.
- ii. Tank method.

The first method consists of culturing earthworms in containers made of plastic and the medium or their bedding (commonly called as vermibeds) can be done in it. Containers in the shape of rectangular boxes or tubes of one foot width, 3 feet length and 2 feet height can be utilized. The shape of the containers can change according to the availability in the area of culturing. These containers with vermibeds must be placed in a proper environment. Either it can be placed in separate thatched roof shed if economically

viable or can be placed in spaces available inside the house itself. Thus depending on the economical status, the place can be selected. The conditions in maintaining the vermibeds are, direct sunlight must be avoided and it must be safe from insects and rodents.

The second method of culturing earthworms is by tank method. By this way multiplication of earthworms can be done in large scale. For this method, cement tanks of dimensions one meter width $\times$ one meter height $\times$ 3-5 meter length can be constructed. These tanks must be constructed inside a thatched roof shed so that direct sunlight and rain can be avoided. The shed can be of any dimensions according to the land available. Thus the length of the tank and the no of tanks to be constructed can also be done accordingly. The shed must be constructed in East-West direction length wise to avoid direct sunlight and preferably open from all sides with unpaved floor with raised ground (atleast 6 inches) to protect the area from flooding during the rains.

2.1.1. Vermibed preparation

The vermibed for both the methods can be prepared using any kind of degradable, non-toxic organic waste like leaf litters, agricultural wastes (Suthar, 2010), shredded newspaper (Updegraff, 1971), cardboard, etc... can be utilized as medium. Wastes from fruit and vegetable market such as potato peels, onion peels, cabbage leaves, carrot and radish leaves, lettuce leaves, moldy bread can also be used (Suthar, 2009). Though organic wastes serve as food for earthworms, they can't be directly implemented for vermiculture. In other words, the worms can't eat the organic matter directly. Thus it must be subjected to partial decomposition and then used as a medium for vermibed.

The organic matters must be shredded and alternate layers of organic matter and mixture of cow dung is spread on the floor of shed constructed for vermiculture. Before the implementation of

organic matter for partial decomposition, care must be taken that, both the organic matter and the cow dung are shade dried. This is because; wet organic matter and cow dung may contain the cyst and larvae of other microorganisms and insects. So if implemented wet, they may grow along with earthworms and do hindrance.

The alternate layers of organic matter and cow dung are arranged in the form of heap and 50% moisture must be maintained. Cover the heap with thatched coconut leaves. This set up must be kept for atleast 30 days. The organic matter, along with cow dung mixture must be turned over with a spade once in a week. The microorganisms which are present in the cow dung slowly degrade the organic matter. After 40 days the process of partial decomposition will be finished and now it can be used as a medium for vermibed.

In the containers, the vermibed must be prepared by mixing the partially decomposed organic waste and cow dung (shade dried and powdered) in 1: 1 ratio. This ratio is recommended by many scientists and has been proved in many literatures also. Nearly 50% of moisture must be maintained in the bed. Earthworms can be introduced after two days. Leave the setup for 45 days. The worms eat the degraded organic matter and convert them into vermicastings. At the same time they also reproduce and increase in number.

Vermibed for tank method can also be prepared in the same way but the partially degraded waste and the cow dung must be added in large quantities into the tank. Earthworms can also be released in large quantities. Water must be sprinkled on the vermibed once or twice in a week according to the humidity prevailing in the atmosphere. Temperature of about 37 degree Celsius must be maintained. If the climate is hot, wet jute bags can be screened on the sides of the tank. By the tank method, earthworms can be reproduced 300 times greater within 45 days or 60 days (depending upon the worms cultured). The reproducing capaci-

ty of exotic worms is greater than the indigenous worms. It is said that eight red worms multiply into 1,500 redworms (*Eisenia fetida*) in six months. These earthworms can be used to prepare vermicompost or for other purposes or can be sold.

The vermibeds must be covered with a Jute mat to protect earthworms from birds and insects. Water is sprinkled on the vermibeds daily according to requirement and season to keep them moist. The waste is turned upside down once in a week. The appearance of black granular crumbly powder on top of vermibeds indicates the casts of earthworm. Addition of more amount of cow dung ensures the fecundity rate of earthworms.

2.1.2. Harvesting of earthworms

The vermibed after 45 days is converted into vermicastings. This indicates that all the organic matter along with cow dung is eaten up by the worms and converted into vermicastings. Now there is no food available for the worms. The content in the container or in the tank must be removed and new beds must be replaced. Before the replacement, the worms must be harvested. The very recent and new technique followed to harvest earthworms is using “fresh cow dung balls”. Fresh cow dung attracts the earthworms very much. Thus balls of fresh cow dung, of 15 to 20 cm in diameter are placed inside the vermibeds. For container method, one ball is enough while for tank method, four to six balls can be used to harvest earthworms.

As soon as the cow dung balls are placed, the earthworms start migrating into the fresh cow dung balls. The cow dung balls were kept for 6 to 8 hours, after which are removed. Thus the earthworms can be harvested and used as inoculums for the new vermibed. The vermicastings in the container or the tank is removed and made into heap. This heap is left undisturbed for a day. Next day, the vermicastings are removed slowly from the outer side with the help of spade, sieved, and packed as biofertilizer. The

small worms (juveniles) present segregate in the centre of the heap and can be collected.

2.2. Application of earthworms

Adult earthworms can be sold as fish bait and the young ones can be used as inoculums for fresh bedding. Many industries like aquaculture and Poultry will buy earthworms for using it as live feed. Business people who are interested in constructing vermicomposting units (house hold, small scale or large scale unit) will be purchasing the earthworms. Earthworms can be sold to new business people who are about to start their vermiculture or composting unit. Worms are also bought by academic institutions for research purposes. Earthworms can be sold to wholesalers who then resell the worms to bait shops, home and organic nurseries, and other users.

3. Vermicomposting

Earthworms facilitate the stabilization of organic wastes because their activity maintains aerobic conditions and ingested solids are converted into discrete odourless casts (Edwards, 1988). Thus the end product of vermicomposting is referred to as "vermicasting" or vermicompost. This is a nutrient rich organic substance that can be added to soil to increase its organic matter content and available nutrients. Vermicomposting is getting enormous importance in the amelioration of severe problems associated with the disposal of large quantities of organic wastes (John Paul, 2005). Earthworms feed on organic matter, in which 5-10% is taken as food intake for their growth and the rest is excreted. Exotic earthworms like Red worms (*Eisenia fetida*) and African worm (*Eudrilus eugeniae*) and Indigenous species like *Lampito mauritti* and *Perianyx excavatus* are proved to be effectively in vermicomposting (Karmegam and Daniel, 2009, Kaur, et al., 2010). Vermicomposting can be done in three ways.

- i. Container method
- ii. Tank method
- iii. Pit method

For all the three methods, the pre-processing of organic matter, i.e. the partial decomposition of organic matter is essential and it can be done as said above for vermiculture.

3.1. Container method

This method of composting is normally followed in small scale vermicomposting units or in houses where vermicomposting is done. The containers and the vermibed preparations can be followed as said for vermiculture. The only difference is the ratio of organic matter and cow dung. For vermiculture, the ratio of 1: 1 must be maintained in order to increase the fecundity rate. Because, the main aim of vermiculture is production of earthworms. While during vermicomposting, the ratio can be altered as 2: 1 or even to 3: 1 according to the availability of cow dung. An advantage of container method over traditional composting, is that they can be kept inside the composting shed during the winter, thus allowing this process to be done all over the year.

3.2. Tank method

Same as for vermiculture, the tank can be constructed and the process of vermicomposting can be done. Thus this method counts for large scale of vermicomposting. The only difference in both is, production of large amount of earthworms is the aim in vermiculture while production of large amount of vermicompost is the aim in vermicomposting. The same type of shed can be constructed for this purpose. The size can according to the availability of organic waste (the shed can be constructed in the same way as said for vermiculture).

3.3. Pit method

A pit of approximately 4m×6m×4m (breadth× length× depth) must be constructed. Vermibed is actually constructed with a layer of loamy soil placed

at the bottom, about 15 to 20 cm thick above a thin layer (5 cm) of broken bricks and coarse sand. Earthworms are introduced into the loamy soil, which the worms will inhabit as their home. 150 earthworms may be introduced into a compost pit of about 2m x 1m x 0.75m, with a vermibed of about 15 to 20 cm thick. Handful lumps of fresh cattle dung are then placed at random over the vermibed. The compost pit is then layered to about 5 cm of dry leaves or preferably chopped hay/straw or agricultural waste biomass or any non-toxic organic waste. This layer of organic waste and cow dung must be repeated till the top of the surface. For the next 30 days the pit is kept moist by watering it whenever necessary. The bed should neither be dry or soggy. The pit may then be covered with coconut or Palmyra leaves or an old jute (gunny) bag to discourage birds. Plastic sheets on the bed are not recommended as they trap heat. All these organic wastes can be turned over or mixed periodically with a pick axe or a spade. If the weather is very dry it should be dampened periodically. Red worms and African worms consume large amounts of organic matter and hence they are recommended for composting. Though exotic earthworm species are used in pit method, the indigenous worms as invade the pit and do their role.

3.4. Precautions

Vermicomposting unit or pit should be protected from direct sun light. To maintain moisture level, spray water on the composting unit as and when required. Large amounts of waste can cause odours and attract dogs or rodents, ant, rat and bird. Thus, preventive measures should be taken like net or anti-insect propellants to avoid them.

3.5. Harvesting Vermicompost

Vermicompost can be harvested following the same method as that of vermiculture, using fresh cow dung balls and heap method. Some consumers are picky

in getting the pure form of vermicompost, i.e. the vermicasts, which are collected on the top of vermibed. At that case, vermicasts can be collected with a spatula once in a week and can be packed and sold. The rate of pure form of vermicastings is also higher when compared to the vermicompost. When large amount of compost are to be harvested, for example the compost obtained from tank method or from pit method, first the worms are harvested with the help of fresh cow dung balls, then the whole material is moved to a plain area or on a plastic sheet and made into a single heap and is exposed to light, scooped, sieved and packed for sales. Earthworms or undecomposed materials if seen any, are collected and returned to the compost pile.

For faster rate of harvesting vermicompost, the original heap is better divided into several smaller pyramids. To enhance the earthworm movement towards the centre of the heap, ball of raw or fresh cow dung can be placed at the centre. Always earthworms have high affinity towards fresh cow dung. Thus after few hours the worms can be easily separated and the compost can be harvested and packed.

3.6. Advantages of vermicompost

- Vermicompost is rich in all essential plant nutrients, hence provide excellent effect on overall plant growth, encourages the growth of new Shoots / leaves
- Vermicompost is free flowing, easy to apply, handle and store and does not have bad odour.
- It improves soil structure, texture, aeration, and water holding capacity.
- Vermicompost is rich in beneficial microorganisms which fix the Nitrogen and Phosphorous in the soil.
- Vermicompost may contain earthworm cocoons from which juveniles may come and increases in population in the soil where applied.

- It neutralizes the soil acidity or alkalinity.
- Vermicompost is free from pathogens, toxic elements, weed seeds etc.
- Vermicompost minimizes the incidence of pest and diseases.
- It contains valuable vitamins, enzymes and hormones like auxins, gibberellins etc.
- The nutrients available in vermicompost (in general) are Organic carbon (9.5 – 17.98%), Nitrogen (0.5 – 1.50%), Phosphorous (0.1 – 0.30%), Potassium (0.15 – 0.56%), Sodium (0.06 – 0.30%), Calcium and Magnesium (22.67 to 47.60 meq/100g), Copper (2 – 9.50 mg kg⁻¹), Iron (2 – 9.30 mg kg⁻¹), Zinc (5.70 – 11.50 mg kg⁻¹) and Sulphur (128 – 548 mg kg⁻¹).

3.7. Storing and packing of vermicompost

Watering is stopped for atleast 5 days at this stage. The first lot of Vermicompost is ready for harvesting after 2-2 ½ months and the subsequent lots can be harvested after every 6 weeks of loading. The vermibed is loaded for the next treatment cycle. The harvested vermicompost should be stored in dark, cool place. It should have minimum 40% moisture. Sunlight should not fall over the composted material. It will lead to loss of moisture and nutrient content. It is advocated that the harvested composted material is openly stored rather than packed in over sac. Packing can be done at the time of selling. If it is stored in open place, periodical sprinkling of water may be done to maintain moisture level and also to maintain beneficial microbial population. If the necessity comes to store the material, laminated over sac is used for packing. This will minimize the moisture evaporation loss. Vermicompost can be stored for one year without loss of its quality, if the moisture is maintained at 40% level.

4. Vermiwash

Vermiwash is the liquid bio-fertilizer collected after the passage of water through a column of worms. It is very useful as a foliar spray. It is a collection of excretory products and excess secretions of earthworms along with micro-nutrients from soil organic molecules. Vermiwash units can be set up in a plastic or iron barrel of 200 litre capacity for a large scale production. While for production in small scale, a plastic bucket of 15 litre capacity is enough.

4.1. Method of preparation

The holding unit, i.e. the plastic barrel must be taken and fixed in a stand or on a high platform. A hole is drilled on one side at the bottom and a vertical limb of a T joint tube is attached in a way that half to one inch of the tube projects inside the barrel. A tap is attached to the end of the horizontal limb and the other end is closed with a dummy nut. The whole set up is mounted on a suitable pedestal. Keeping the tap open, a layer of broken bricks or pebbles is filled up to 25-30cm inside the barrel. Water is made to flow through this layer, followed by 20-30 cm layer of coarse sand. This forms the basic filtering unit. Over this a 60-75 cm layer of good loamy soil along with organic waste and cow dung is kept moistened. In this layer earthworms like, *E. fetida*, *E. eugeniae* and *lumbricus terrestris* may be introduced. Cattle dung pats and hay are placed on top of this layer of soil for mulching purpose. The unit is moistened every day. For two days after the introduction of earthworms the tap must be kept closed. After two days, open the tap, about 30 – 40 litres of vermiwash drains out.

Similar setup can also be made in bucket for production in small scale. The layer of organic matter here last for 30-45 cm thickness and the mulching of 2-3 cm thicknesses must be spread to prevent evaporation. Spray water regularly for 7-8 days for both of the setup so that 60% of moisture is maintained. Introduce 1000-2000 numbers of earthworms for the bar-

rel setup while 100-200 earthworms is enough for a bucket. Keep a pot at the bottom of the stop cork of the bucket so that waterfalls drop by drop. Every day 30-40 litres in barrel and about 3-4 liters of vermiwash in the bucket setup can be collected.

As the tap is closed and water is sprinkled on top of the unit, the water slowly percolates through the compost carrying with it nutrients through the filter unit. When the tap is opened after two days, vermiwash is collected, which is sprayed on plants as a foliar spray. The vermicasts formed on the surface of the unit may also be collected periodically.

4.2. Application

The vermiwash may be diluted with water in 1:1 ratio or it may be diluted with 10 per cent cow's urine, which is an effective growth tonic and pesticide. Mix 1 liter of vermiwash with 7-10 liters of water and spray the solution on the leaf (upper lower side) in the evening at the growing crop. Mix 1 liter of vermiwash with 1 litre of cow urine and then add 10 liters of water and mix thoroughly and keep it over night before spraying. 50-60 litres of such solution can be sprayed in one hectare of land to control various crop diseases.

5. Perspectives

As a processing system, the vermicomposting of organic waste is very simple. Worms ingest the waste material - break it up in their rudimentary gizzards, consume the digestible, putrefiable portion and then excrete a stable, humus-like material that can be immediately marketed and has a variety of documented benefits to the consumer. Vermitechnology is a promising technique that has shown its potential in certain challenging areas like augmentation of food production, waste recycling, management of solid wastes etc. In most of the countries, soil pollution is increasing due to accumulation of organic wastes and on the other side there

is shortage of organic manure. Organic waste could be converted into vermicompost using Vermitechnology to increase the fertility and productivity of the agricultural land and to produce nutritive and safe food. Hence we need to use and promote this ecofriendly technology for the sustainability of agriculture and environment.

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Role of Biotechnology in Food Authentication

Shobana Manoharan¹, Raghavan Kuppu¹ and Ramesh Uthandakalaipandian^{2,*}

¹Department of Molecular Biology, School of Biological Sciences, Madurai Kamaraj University, Madurai - 625021, Tamil Nadu, India; ²Assistant Professor, Department of Molecular Biology, School of Biological Sciences, Madurai Kamaraj University, Madurai - 625021, Tamil Nadu, India; *Correspondence: ramesh.biological@mkuniversity.org; Tel: +91-9489014892

Abstract: Food is the most essential component of life for survival. The quality of food products is essentially to be authenticated as they are closely related to the health of human beings. “Foodomics”, is a concept to utilize technology for improvement of food and nutrition. Food products are authenticated in benefit of both consumers as well as commercial traders. Food authentication is done by analytical techniques like chromatography, Fourier Transform Infrared Spectroscopy (FTIR), Nuclear Magnetic Resonance Spectroscopy (NMR), Gas Chromatography - Mass Spectroscopy (GC-MS) and Liquid Chromatography - Mass Spectroscopy (LC-MS). Plant or animal based food products are widely authenticated for their origin, species nomenclature using DNA barcodes - a genomics based approach. The protein molecules of food products serve as key molecules for authentication using proteomics approach such as 2-dimensional gel electrophoresis and 2-dimensional difference gel electrophoresis (DIGE). Thus, food authentication is mandatory to substantiate the geographical origin, species nomenclature, food composition, genetic modification of a food product that reaches to the hands of consumers in the food markets. Thus, the development of rapid, novel food authentication methods to validate sea food products, Genetically Modified (GM) food products based on biotechnological approaches helps to provide quality assured food products to the human community. This review briefs about the various techniques employed in food authentication, their advantages and applications in food biology.

Keywords: Analytical techniques; food authentication; genomics; proteomics

1. Introduction

Nowadays wide range of food products are available from various countries to the consumers but the quality of the food consumed is of great concern as they are directly linked to human health. “Foodomics” is a new term coined at the International Conference, Cesena (2009), Italy (foodomics.eu) which deals with the application of the recent ‘omics’ technology to promote the field of food and nutrition. Foodomics is thus defined as, “a new approach to food and nutrition that studies the food domain as a whole with the nutrition domain to reach the main

objective, the optimization of human health and well-being” (Capozzi and Bordoni, 2013). According to Hippocrates ‘Let food be thy medicine and medicine be thy food’; hence, the ratification of food components becomes vital to shield human health. Development of quality assurance methods to authenticate the food products based on their geographical origin, composition, originality, certification of their species in case of animal or plant derived products are of prodigious attention from both consumer and commercial trader’s opinion. The need for food authentication and its application differs from country to country. For ex-

ample, geographic origin of the food is a principal authenticating criterion to be mandatorily confirmed in Europe, numerous schemes for food quality analysis like PDO (Protected Designation of Origin), PGI (Protected Geographical Indication) and TSG (Traditional Specialities Guaranteed) are also followed by them in order to look after the conventional production methods (Drivelos and Georgiou, 2012). There are a huge number of valid reports till date stating about the adulterants, substitutes and confused species in herbal formulations as like the recounted instances of Chinese herbs that caused severe intoxications and even deaths due to adulterants or substitutes (But, 1994). Thus, food authentication is indispensable, in earlier days it was carried out based on analytical chemistry techniques. Conversely, in recent days it is accomplished by various other methods like genomics, proteomics and metabolomics for the reason that food authentication being a multi-disciplinary research that utilizes data from biology, chemistry, chemometrics and bioinformatics (Ibanez *et al.*, 2013; Moore *et al.*, 2012).

2. Food authentication

Food labelling is a common practice to be followed in order to approve the originality of food products bought in hand to the consumers. Food authentication is the process of confirmation a food product for its originality as described in their labels (Figure 1). This process has grabbed wide attention because of the up-surging perception among the people about food quality and safety (Danezis *et al.*, 2016). Food authentication is mainly done at the following circumstances,

- i. Validation of imported traditional foods,
- ii. Identification of Genetically Modified Products (GMPs) from unauthorised GMP's,
- iii. Identification on adulterants or substitutes,
- iv. Corroboration of medicinally important plant species that may contain confused species,
- v. Verification of Geographical origin of plant or animal derived food products (Figure 2).

3. Chemometric methods

Chemometric methods comprises of chromatographic and spectroscopic methods. Chromatographic analysis sepa-

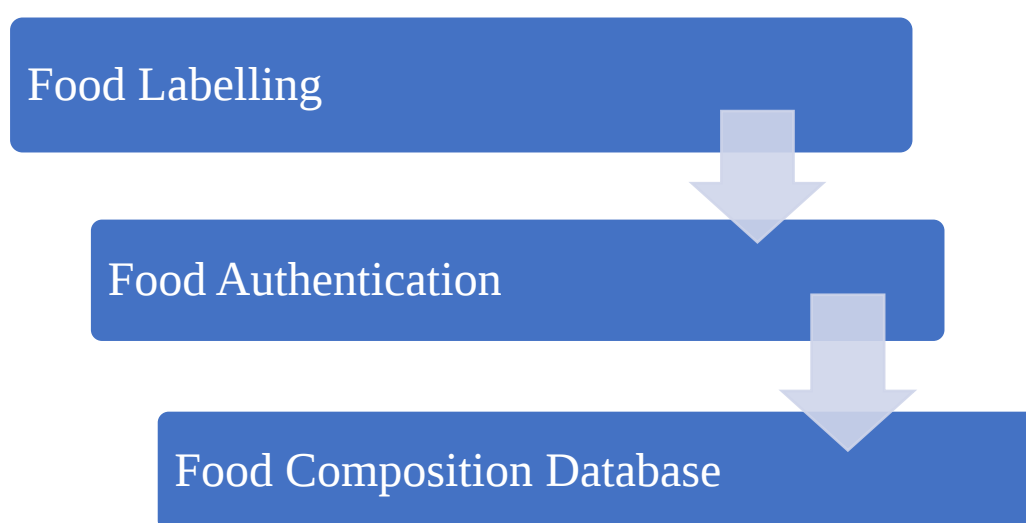


Figure 1: Important steps involved in developing a database for food industry.

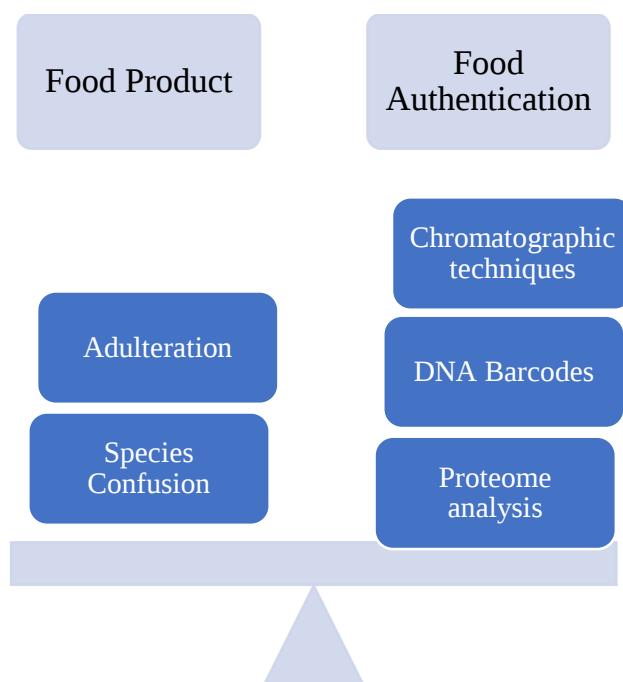


Figure 2: Importance of food authentication.

rates analogous chemical compounds in food products. Food is composed of proteins, lipids, carbohydrates, phytochemicals and many other small molecules such as food additives, colourants and preservatives. In general, these compounds are chemically distinct based on their molecular weight, polarity, charge etc., Development of fingerprint pattern using chromatography (High Performance Liquid Chromatography - HPLC) to differentiate food free from adulterants or constituents succours in food labelling (Cserhati *et al.*, 2005; Reinholds *et al.*, 2015; Georgiou and Danezis, 2015).

Fourier Transform Infrared Spectroscopy (FTIR) analysis can be done for the functional assessment of nominal frequency wave number obtained from the metabolites studied. They identify the presence of fatty acids and other metabolites of the food and help in development of the FTIR fingerprint region which can serve as a unique, preliminary tool to indicate the nutritional index of the food. Further analysis of metal concentrations can be done using Atomic Absorption Spectra (AAS) or Inductively Coupled Plasma Mass Spectroscopy (ICP - MS). Fatty acids and Protein analysis using LC - MS and GC - MS techniques respective-

ly facilitate the identification of individual molecular components from which unique marker compounds for a food may be identified. Nuclear Magnetic Resonance Spectroscopy (NMR) analysis is a fast and more reliable technique to profile the complete metabolome of a food. The peaks pertaining to the functional groups are analysed and unique peaks are used for differentiating the food in terms of quality (Cozzolino, 2012; Longobardi *et al.*, 2013; Hohmann *et al.*, 2015). Proton Transfer Reaction Mass Spectroscopy are used for analysis of volatile organic compounds, reports based on these techniques distinguish organically grown tomatoes from conventional ones (Hohmann *et al.*, 2015). In few instances, highly complex food products are difficult to be differentiated using HPLC, FTIR or NMR at such rationale Gas Chromatography (GC) or Liquid Chromatography (LC) coupled to Mass Spectrometry (MS), have flourished as better food authentication tools (Figure 3). Few applications of chemometric methods are sated below,

- i. Authentication of wild, farmed fish food species (Capuano *et al.*, 2012),
- ii. Fatty acid content confirmation in animal and plant ex

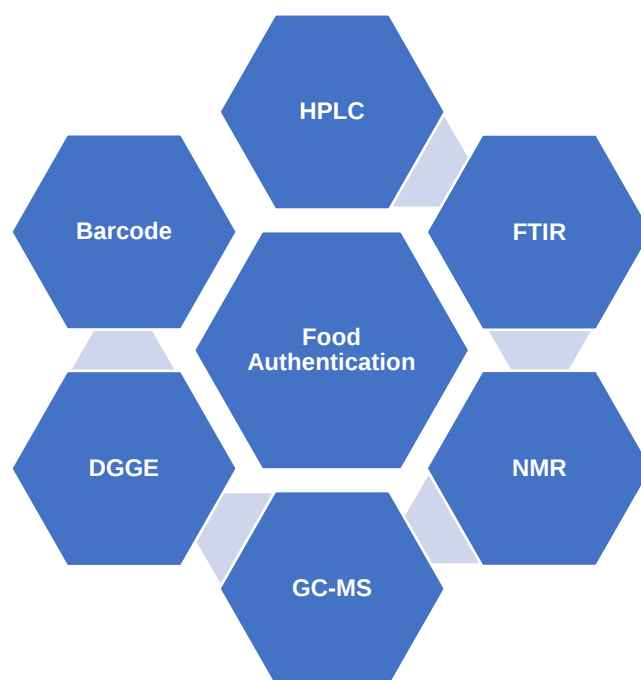


Figure 3: Techniques involved in food authentication.

- traced oils (Yang *et al.*, 2013),
- iii. Validation of mineral content in eggs (Giannenas *et al.*, 2009).

4. Genomic methods

This approach involves the use of genetic material, Deoxyribonucleic Acids (DNA) either as complete genome for evaluation or amplification of signature DNA sequences to authenticate a food product. Molecular techniques such as Random Amplified Polymorphic DNA (RAPD), Restriction Fragment Length Polymorphism (RFLP), Inter Simple Sequence Repeats (ISSR), Simple Sequence Repeats (SSR), Sequence Characterized Amplified Region (SCAR) use the genome variation in certification of food products (Ali *et al.*, 2014). Meta DNA barcoding, *de novo* sequencing and Next Generation Sequencing are some of the techniques that lead to the high throughput sequencing of entire genome. Denaturing Gradient Gel Electrophoresis (DGGE) is used in microbial food authorization such as cheese, curd, probiotic yoghurts and other fermented dairy products (Arcuri *et al.*, 2013). Commercial herbal

formulations are mostly in processed or powdered form, in those cases mini DNA barcodes are employed for authentication. And most of the food products of animal and plant origin are in processed forms where the food source cannot be verified by other chemometric methods and in such circumstances DNA barcodes play a vital role in validation of plant or animal species used in manufacturing of a food product. DNA barcodes are short, conserved sequences that are employed in molecular taxonomy classification of plants and animal's species. Cytochrome Oxidase I [CO I] gene of mitochondrial (mt) DNA is a universally acceded molecular marker used for DNA barcoding in animals (Hebert *et al.*, 2003). In plants, maturase K (mat K) and RubisCO L (Ribulose 1, 5 bisphosphate carboxylase / oxygenase) gene abet in barcoding (Janzen *et al.*, 2009).

5. Proteomic methods

Food proteomics research helps in analysis of individual marker protein components from mixture of proteins using Polyacrylamide gel electrophoresis (1D or 2D) along with LC - MS for protein characterisation. Characterisation of

Table 1: Applications of food authentication

No	Food Product	Authentication Method	Source
1.	Olive Oil	GC - MS	Yang <i>et al.</i> , 2013
2.	Eggs	ICP - MS	Giannenas <i>et al.</i> , 2009
3.	Salmon	¹ H NMR	Capuano <i>et al.</i> , 2012
4.	Tomatoes	Proton Mass Spectroscopy	Hohmann <i>et al.</i> , 2015
5.	Italian Sweet Cherry	NMR	Longobardi <i>et al.</i> , 2013
6.	<i>Nigella sativa</i> seed oil	FTIR	Nurrulhidayah <i>et al.</i> , 2011
7.	Cheese	16s rDNA PCR and DGGE	Arcuri <i>et al.</i> , 2013

genetically modified products, soy proteins in foods and dairy products are done based on proteomic analysis (Gallardo *et al.*, 2013). This proteomics based approach is of wide importance and applications in food industry as they accurately pin point the difference between original and adulterated food products.

6. Immunological methods

Immunological methods rely on the binding specificity of the antibodies designed to an atypical antigen *i.e.*, allergens, toxins etc. in food by use of Enzyme Linked Immuno Sorbent Assay (ELISA) (Asensio *et al.*, 2008). For instance, they are used to spot the presence of potential fish allergen parvalbumin (Gajewski and Hsieh, 2009) in seafood industry. These techniques rely on specificity and also have an added advantage of validating a large number of samples with high precision in a short span of time.

7. Perspectives

Food authentication not only ensures food quality but also assures human health. Emergence of new research fields such as transgenics, foodomics, nutrigenomics has resulted in wide range of neoteric products that need to be validated for substitutes prior to their distribution in commercial markets for edible purpose. Some of the practical applications of food authentication based on the above-mentioned techniques are tabulated in Table 1. Construction of food authentication

databases will be fruitful for high throughput validation of food products.

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Management Strategies against Tiny Tigers for Sustainable Development of Agriculture

Viswa Venkat Gantait*

Zoological Survey of India, M-Block, New Alipore, Kolkata-700053, West Bengal, India;

*Correspondence: v.gantait@rediffmail.com; Tel: 09433463555

Abstract: Amongst different pests of agriculture, the plant parasitic nematodes are considered the worst one because of devastations they cause to the crops. Due to their microscopic nature, they are the hidden enemies of soil and crop damages caused by them have not been fully formulated. For sustainable development of agriculture and to stop the yield losses caused by these tiny pests of crops, potential control measures should be explored and adopted against them. By following various physical, chemical, biological and botanical methods, some of cultural practices and also by regulatory methods nematode infestation in agricultural fields must be stopped or checked at certain level. This will help to enhance crop production, which will ultimately be helpful to improve the gross national product (GNP) of country and agricultural sustainability. This article provides an overview of management strategies which could be used against plant parasitic nematodes to boost the sustainable development of agriculture.

Keywords: Agriculture; control measure; plant parasitic nematode; sustainable development

1. Introduction

1.1. What are tiny tigers?

Tiny tigers! How funny the term is? Tiger is one of the most ferocious carnivores of the world. Not a joke, the nematodes are now treated as tiny tigers in crop fields. Even, these are more harmful than tigers, as far as agriculture is concerned. They represent one of the most abundant groups and probably the second largest one in the animal kingdom, immediately behind the arthropods (Hugot *et al.*, 2001).

Nematodes represent sharply differentiated primitive group of invertebrates, commonly known as round worms, thread worms or eelworms, distinctly different from segmented worms like earthworms and flatworms. Simply, these are also called 'nemas'. These are multicellular, vermiform, triploblastic, pseudocoelomate, bilaterally symmetrical, unsegmented animal, possessed between

the phylum Platyhelminthes and Annelida in the animal kingdom. They generally have a cylindrical body while a few may be fusiform, saccate or kidney-shaped. Those are characterized by having a body cavity, complete digestive tract, well developed reproductive system, excretory and nervous system; but lacking circulatory and respiratory system. Most of them are microscopic in size, but may be seen with naked eyes.

1.2. Where we can find them?

Nematodes are highly diverse in their habitats ranging from Himalayan peak to the sea floor, from Arctic to Antarctic (Lal, 1998). They can withstand extreme adverse environmental conditions and may be found in Polar Regions to tropics. They occur almost everywhere on the earth like in ocean, river, lake, pond, estuary, island, soil, hill and rocks, desert hot springs etc. Interestingly, their habitat is unsurpassed by any other meta-

zoan invertebrate group, because they are found in all types of habitats (Bohra and Baqri, 1997). They are so numerous that if everything on the earth were to disappear except the nematodes, the outlines of habitats would still be dimly visible; the mountains, lakes and oceans, the plants and the animals would all be outlined by the nematodes present (Cobb, 1914).

Based on the habitat, the total nematode population can be categorized into three groups: marine, animal-parasitic, soil and freshwater nematodes (Ayoub, 1980). The marine nematodes constitute about 50% of the total nematode population, where as animal-parasitic nematodes make up only 15% of the known nematode species. The soil and freshwater nematodes can be spitted into two finer divisions: free-living and plant-parasitic. Free living nematodes comprising about 25% of the total nematode population. The plant-parasitic nematodes constitute only 10% of the total nematodes (Figure 1).

According to Crofton (1966) most of the soil holds about 90% nematodes at top six inches. (Nicholas, 1984) opined that the soil nematodes are usually most abundant near the soil surface, with the majority within the top 10 cm, though some may be found much deeper. He also stated that, populations are much denser in the zones, rich in organic matter or

where fine plant roots are concentrated; adequate soil moisture and oxygen also favours dense populations of nematodes.

1.3. What they feed?

Nematodes are microphagous, mycetophagous, saprophytic, phytoparasitic, predatory, carnivorous and even cannibalistic in nature. Most of them feed on bacteria, fungi, other microorganisms and decaying matter. They can parasitize plants, different animals including man and even other nematodes also. Depending on the diverse diets Yeates *et al.* (1993) categorized nematodes into eight feeding groups: plant feeders, algal feeders, hyphal feeders, substrate ingesters, unicellular eukaryote feeders, bacterial feeders, predators and omnivorous.

2. Important roles in agricultural aspects

Plant parasitic and soil-inhabiting nematodes have been known for their virulence causing significant loss to agricultural and horticultural crops (Bohra and Baqri, 2004). Several species have come to be recognized as useful predators in the control of different insects and nematodes also. The possibility of using nematodes for control of different plant parasitic nematodes was first suggested by Cobb (1917). The role of predatory nematodes

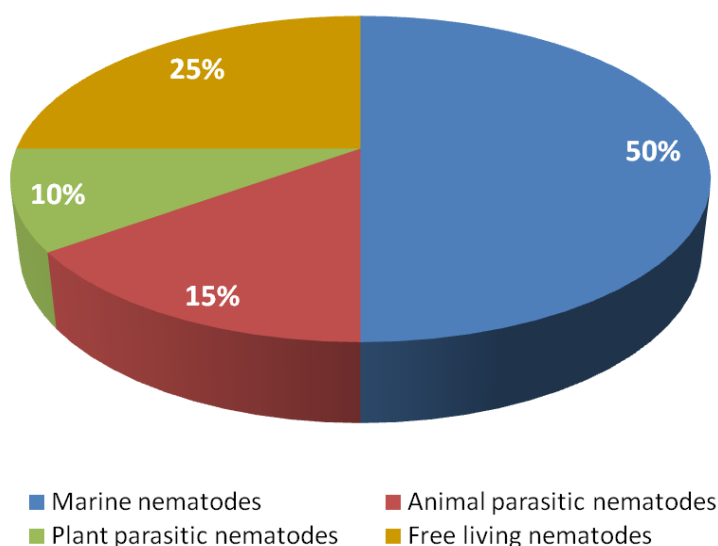


Figure 1: Pie diagram showing the different groups of nematodes.

in biological control of plant parasitic nematodes was described by Jairajpuri *et al.* (1990). They regulate microbial biomass and nitrogen mineralization by killing and feeding on nematodes and other microorganisms that pass from bottom to top trophic levels in an ecosystem (Wardle and Yeates, 1993). Some species are known to play a vector role in transmitting so many soil-borne bacterial, fungal as well as viral pathogens to their hosts (Jairajpuri and Ahmad, 1992). The innocuous nematodes, those have no concern to the farmer or gardener; those feed on bacteria, fungi, algae and even other nematodes, play an important role in controlling soil nutrient cycling (Tahseen, 2006). Each species possibly plays a significant role in the ecosystem inhabits and undoubtedly has a major role in maintaining the natural ecological balance. Nematodes are intimately involved in many parts of the soil ecosystem, so they can be used as bioindicators of sustainability for soils. Because of their ubiquity and diversity, nematodes are used in measuring the impact of various perturbations on ecosystems, such as pollution, organic enrichment and physical disturbance (Tahseen, 2006).

2.1. How they depress crop yields

Plant parasitic nematodes depress crop yields by the following important ways.

- They feed on plant parts and deprive the host of its nutrients.
- During feeding they cause mechanical injury to different parts of plants and their feeding sites serve as entry-points of other pathogenic fungi and bacteria.
- Act as vectors of different fungal, bacterial and viral pathogens of plants.
- They can secrete various enzymes in the plant tissues during feeding and the effected plants show abnormal growth responses to these secretions/excretions like hypertrophy.

- They can also act as modifiers of host substrates and render them more susceptible to other plant pathogens.

3. Important symptoms of their attack

Being protected under soil and having microscopic size, nematodes are practically the hidden enemies of crops. Symptoms of their attacking are not striking in most cases and therefore overlooked. The important symptoms may be tabulated as follows (Table 1).

4. Common names of some important nematodes

The plant parasitic nematodes belonging to the order Dorylaimida are migratory root ectoparasitic in nature. The genera, *Trichodorus* and *Paratrichodorus* under the family Trichodoridae browse along the root surface of plant. On the other hand, genera like *Longidorus*, *Paralongidorus* and *Xiphinema* feed for longer period at specific sites of deeper root tissues. Apart from causing direct minor or major root damages, these nematodes are of great economic importance as vectors of nearly 22 soil-borne plant viruses. All the Tylenchids i.e. the nematodes belonging to the order Tylenchida are totally plant parasites. They are ectoparasitic, semi-endoparasitic or endoparasitic in nature. Most of the endoparasitic nematodes form root-galls and leaf-galls of plants. The common names of some important plant parasitic nematodes are as follows.

5. Management strategies

For sustainable development of agriculture, the adverse effects of nematodes have to be eradicated or minimized. For this purpose different control measures should be adopted against them. The first and foremost effort is to reduce increasing populations of these hidden enemies of crops, under the soil. The di-

Table 1: Symptoms of nematode infestation

Symptoms	Caused by the species
Produced by above-ground feeders	
• Crinkled and distorted stems and foliages	<i>Anguina spp.</i>
• Dead and devitalized buds	<i>Aphelenchoides spp.</i>
• Leaf lesions	<i>Aphelenchoides besseyi</i>
• Leaf and seed galls	<i>Anguina spp.</i>
• Necrosis and discoloration	<i>Rhadinaphelenchus spp.</i>
Produced by below-ground feeders	
• Poor growth, stunting patchiness, discolored foliage, wilting etc.	Most of the species
• Root galls	<i>Meloidogyne spp.</i> , <i>Nacobbus spp.</i> , <i>Ditylenchus spp.</i> , <i>Xiphinema spp.</i> etc.
• Root lesions	<i>Pratylenchus spp.</i> , <i>Radopholus spp.</i>
• Reduced root system	<i>Trichodorus spp.</i> , <i>Belonolaimus spp.</i>
• Rots of fleshy plants	<i>Ditylenchus spp.</i>

rect and indirect benefits to control these tiny tigers of agricultural fields improved plant health, thereby reducing their changes of suffering from nematode diseases and also increased the plants ability to withstand adverse growing conditions, resulted improvement of crop production. Principal strategies for nematode management are cultural practices, physical, biological, botanical, chemical and regulatory methods.

6. Cultural Practices

To control nematode pests, cultural practices are the most effective and economical means which can be achieved by crop rotation, fallowing, deep summer ploughing, water flooding, growing antagonistic or trap crops, removal of infected plant debris, selection of healthy planting materials, application of organic manures and fertilizers etc.

Some species of nematodes are able to feed and multiply on certain crops, but not on others. The crop plants on which they cannot feed and abundantly reproduce are called non-host. By crop rotation system, the crops to be grown in between the susceptible host crops should be immune or resistant to nematodes or at least non-host plants which help to eradicate or minimize the nematodes problems.

Fallowing is a very common practice and cheaper way to minimize the nematode problems by keeping the lands from all vegetations for a certain period. Complete fallow without allowing any plant or weed to grow invariably ensures the parasitic nematodes will have no host to feed. Thus, those are deprived of food and killed by the solar heat and soil desiccation.

Deep summer ploughing involves exposure of soils to solar heat and desiccation, helps to kill the nematode pests along with other pathogens. For enhancement of the efficacy of solar heating, polyethylene mulching of moist soil during hottest period is being advocated. It considered as a very effective and good control measure against nematode pests and can be used with other cultural practices like crop rotation, green manuring, inter cropping as well as with nematicides etc. (Mathur *et al.*, 1987).

In the field where there is enormous availability of water and nematode infested area is uniformly leveled, flooding can be adopted as a routine practice and very effective measure. Under submerged condition, chemicals lethal like hydrogen sulphide and ammonia to these noxious pests are released. Asphyxiation and microbial decomposition products

due to anaerobic condition help to kill the nematodes.

The crops like mustard, marigold, neem etc. are treated as antagonistic crops, those have chemicals or alkaloids as root exudates which repel or suppress the plant parasitic nematodes. These plants can be grown along with main crop or may be included in crop rotation.

The basic concept with trap cropping system is that two crops are grown in the field, out of which one crop is highly susceptible to nematode. When nematodes attack such crop, very carefully it should be removed and destroyed or burnt totally. Thus, the main crop escapes from nematode infestation. Cowpea is a very good example of such susceptible crop for destroying root-knot nematodes.

Early detection of infected plants, immediate removal and destruction of those helps to reduce the spreading of nematodes in the field. Selection of nematode-free healthy planting materials or plantation of nematode-resistant varieties is also a very effective method to avoid nematode infestation in field.

Soil treatments with organic manures, green crop residues or green leaf manures, farm yard or poultry manures, oil cakes, and oils of neem, karanja, castor etc. significantly checked nematode population. The use of such materials also encourages the development of nematode-antagonistic microbes and predacious nematodes also those help to control nematode infestation in agricultural fields.

6.1. Physical methods

The hot water treatment, hot water drenching, rabbing with slow burning materials, soil solarization, electrical soil heating, washing and cleaning of seed etc. are the most effective physical methods to control nematodes infestations to crops.

For denamatization, rhizomes, bulbs, corns, tubers and fleshy roots of plantations and also other planting materials are submerged into hot water for certain periods. Prior to plating the seed ma-

terials like banana corns, onion bulbs, tuber seeds and roots of seeding can be dipped in 5-55°C hot water for about 10 minutes.

The most primitive way of heating the soil on large scale is by putting a fire over it. Burning a layer of dry leaves on soil killed nematode pests. Rabbing of bajara husk, paddy husk, wheat straw etc. become most effective against nematodes. Burning materials outside and incorporating ash into the soil surface satisfaction nematode kill.

Soil solarization is the most recent method for control of plant parasitic nematodes (Sharma & Trivedi, 1991). The technique consists of covering the moist soil with a good quantity, clear and transparent plastic film during the period of intense sunshine and increasing substantial soil temperature over the non-solarized soil. Increased soil temperature coupled with soil moisture invariably result the significant reduction in population densities of phytoparasitic nematodes. The nematode pests of crops are effectively controlled by this method but it is restricted to summer season and only in the tropical and subtropical region of the world.

Careful washing of tubers, bulb and other planting materials prevent this nematode infestation in new planting fields. Modern mechanical seed cleaning methods have been developed to remove the seed galls to form normal healthy seeds. Sanitation, the use of clean tools and equipments in field also prevent nematodes infestation. Soil amendments and frequent irrigation can also help to reduce nematode-damage of crops.

6.2. Biological methods

Biological control method of nematodes include the use of predaceous and parasitic organisms such as fungi, bacteria, protozoans, viruses, nematodes, tardigrades, collembolans, mites etc, even antagonistic higher plants also. This method, in fact, should be considered a skillful manipulation of the biosphere

against nematodes pest of agricultural fields for achieving maximum benefits. There are three types of components of biological control of nematodes.

Natural: Where the agent are already present at levels to be sufficient for suppression of nematode development.

Induced: The agents are already present in the soil and only their activities are stimulated by modifying the environment or by applying inciters.

Introduced: The agents are applied by man from outside.

There are more than 50 species of predaceous fungi which have the capacity to kill nematodes in agricultural field (Jain, 2003). These fungi capture nematodes by traps, mechanical traps and constricting rings.

There are several reports of bacteria, present inside the nematode body. *Pasteuria penetrans* has been described as potential biological agent against nematodes. They prevent reproduction and eventually kill the root-knot nematodes and many other species. Some rhizospheric bacteria like *Azotobacter chroococcum*, *Azospirillum lipoferum*, and some *Pseudomonas* spp. have found to be promising in reducing nematode population. Root-knot nematode larvae infected with viruses were observed to exhibit sluggishness.

6.3. Botanical methods

Due to their facile biodegradability, selective toxicity only to target pests,

safety to non-target organisms and the environment as a whole and renewable nature, the botanical pesticides offer alternate strategy to the prevalence use of synthetic nematicides (Mishra, 1998). Indiscriminate use of chemical pesticides to control nematode pest in agriculture give rise to serious problems like food contamination, adverse effects on non-target organisms and environment, as well as development of pesticidal resistance in many nematode pests. For this reason, the use of bio-pesticides of botanical origin for the management of plant parasitic nematodes has been increased presently. Different parts of botanicals directly, the extracts of botanical parts or the product of botanicals are used for nematode management.

Parts of different plants having nematicidal value are used directly against phytonematodes, infesting various crops (Table 2). Chopped leaves of pineapple, karanja and neem leaves etc. could be significantly reduced the root-knot, reniform and other nematodes also. Various parts of *Crotalaria*, marigold, Kentucky blue grass etc. in powdered form also reduced nematode population. Chopped castor leaves, Subabool leaves prevent gall nematodes. Chopped shots of latex-bearing plants significantly suppressed the population build up of reniform and root-knot nematodes.

Table 2: Common names of some important phytonematodes

No.	Genera/Species	Common names
1.	<i>A. fragariae</i>	Spring dwarf nematode
2.	<i>Anguina</i> spp.	Seed gall, Leaf gall nematodes
3.	<i>Anguina tritici</i>	Ear-cockle nematode, Wheat gall nematode.
4.	<i>Aphelenchoides besseyi</i>	Rice white tip nematode, White tip nematode
5.	<i>Aphelenchoides ritzemabosi</i>	Chrysanthemum foliar nematode
6.	<i>Aphelenchoides</i> spp.	Bud and leaf nematodes, foliar nematodes
7.	<i>Belonolaimus</i> spp.	Sting nematodes
8.	<i>Belonolaimus gracilis</i>	Pine sting nematode
9.	<i>Cacopaurus</i> spp.	Sessile nematodes
10.	<i>Criconema</i> spp.	Spine nematodes
11.	<i>Criconemoides citri</i>	Citrus ring nematode
12.	<i>Criconemoides</i> spp.	Ring nematodes

Table 2: Continued...

13.	<i>Ditylenchus angustus</i>	Rice nematode
14.	<i>Ditylenchus destructor</i>	Potato root nematode, Potato tuber nematode, Iris nematode
15.	<i>Ditylenchus dipsaci</i>	Stem nematode, Tulip root nematode, Bulb nematode
16.	<i>Ditylenchus myceliophagus</i>	Mushroom spawn nematode
17.	<i>Dolichodorus</i> spp.	Awl nematodes
18.	<i>Dorylaimus</i> spp.	Spear nematodes
19.	<i>Globodera rostochiensis</i>	Golden nematode of potato
20.	<i>Globodera</i> spp.	Cyst nematode
21.	<i>Helicotylenchus</i> spp.	Spiral nematodes
22.	<i>Hemicriconemoides</i> spp.	Sheathoid nematodes
23.	<i>Hemicycliophora</i> spp.	Sheath nematodes
24.	<i>Heterodera avenae</i>	Great root nematode, Cereal nematodes
25.	<i>Heterodera cruciferae</i>	Cabbage cyst nematode
26.	<i>Heterodera glycines</i>	Soybean cyst nematode
27.	<i>Heterodera goettingiana</i>	Pea cyst nematode, Pea root nematode, Alfalfa root nematode
28.	<i>Heterodera schachtii</i>	Sugar beet nematode
29.	<i>Heterodera</i> spp.	Cyst-forming nematodes
30.	<i>Hirschmanniella oryzae</i>	Rice root nematode
31.	<i>Hoplolaimus</i> spp.	Lance nematode, Spear nematode
32.	<i>Longidorus</i> spp.	Needle nematode
33.	<i>Meloidodera</i> spp.	Cystoid nematode
34.	<i>Meloidogyne arenaria</i>	Peanut root knot nematode
35.	<i>Meloidogyne brevicauda</i>	Indian root knot nematode
36.	<i>Meloidogyne exigua</i>	Coffee root knot nematode, Brazilian root knot nematode
37.	<i>Meloidogyne incognita</i>	Southern root knot nematode
38.	<i>Meloidogyne javanica</i>	Javanese root knot nematode
39.	<i>Meloidogyne</i> spp.	Root knot nematodes, Root-gall nematodes
40.	<i>Nacobbus</i> spp.	False root knot nematodes
41.	<i>Paratylenchus</i> spp.	Pin nematodes
42.	<i>Pratylenchus</i> spp.	Root lesions nematodes, Meadow nematodes
43.	<i>Radopholus similis</i>	Burrowing nematode
44.	<i>Rhadinaphelenchus cocophilus</i>	Coconut palm nematode, Red ring nematode
45.	<i>Rotylenchulus reniformis</i>	Reniform nematode
46.	<i>Rotylenchus</i> spp.	Spiral nematodes
47.	<i>Trichodorus</i> spp.	Stubby root nematodes
48.	<i>Tylenchorhynchus claytoni</i>	Stunt nematode, Teaselate stylet nematode
49.	<i>Tylenchorhynchus martini</i>	Sugarcane stylet nematode
50.	<i>Tylenchorhynchus</i> spp.	Stunt nematode, Stylet nematode.
51.	<i>Tylenchulus semipenetrans</i>	Citrus root nematode
52.	<i>Xiphenema</i> spp.	Dagger nematodes

Certain botanicals in the form of aqueous extracts of various parts have great potential against nematodes, the aqueous extracts of fresh neem leaves; fruit skin of *Citrus reticulata* Blanco and *Momordica*

charantia L.; leaves of *Ageratum conizoides* L., *Anacardium occidentale* L., *Argemone mexicana* L., *Datura stramonium* L. etc.; aqueous root extract of *Ocimum sanctum* L.; seed extracts of

Vernonia anthelmintica Wild, *Holarrhea antidysenterica* Wall; bulb extracts of *Allium sativum* L. and many other plant extracts have potentiality to prevent nematode infestation in agricultural fields.

Different plant products like oil seed cakes, oils, seeds, and various other formulations are extensively used for the management of plant parasitic nematodes.

6.4. Chemical methods

The chemicals those are used for controlling nematodes are the nematocides. These are the soil fumigants, applied to the soil and diffuse through the soil as gas and acted against nematodes. The use of nematicides for the management of plant parasitic nematodes in agriculture becomes essential when other methods are unable to protect the crops from these pests, or spreading of nematodes is so high in the field. Before planting, the nematocidal application in the field in proper doses resulted in nematode-free rhizosphere, healthy root system, efficient use of minerals, moisture and also reduces the chances of invasion of other harmful soil microorganism. Kuhn (1881) first used chemical (CS_2) against *Heterodera schachtii* in Germany. The discovery of DD-mixture in 1943, EDB in 1945 and DBCP in 1954 played remarkable role in demonstrating the nematode damage and crop losses. The use of methylisothiocyanate, precursor compounds like daromet, methamsodium, methylisothiocyanate mixture like vorlex etc. also help in controlling nematodes. The non-volatile nematicides like fen-sulphothion, aldicarb, carbofuran, ethoprop etc. are also very promisable nematocides. But the use of nematicides is a costly proposition and creates toxic hazards and environmental pollution. Few of them like DBCP, MBr, aldicarb etc. have been banned already. The use of nematocides is not so popular in agriculture except in few cases where drastic spreading of nematodes occurs in the field.

6.5. Regulatory methods

Quarantine principles are traditionally employed to restrict the movement of infected plant materials and contaminated soil into a state or country. Many serious plant parasitic nematodes spread from one country to another and from one state to other. The potato cyst nematode, *Globodera rostochiensis* spread from Peru to almost whole of Europe and UK through seed potatoes and gunny bags. The stem and bulb nematode, *Ditylenchus dipsaci* got introduced in southern parts of Sweden also through seeds. For this reason, plant quarantine has been introduced at state, national and international levels as a legal restriction to check the spreading of nematode pest. Regulatory control of pests and diseases is the legal enforcement of measures to prevent them from spreading. Strict regulations have been made against *G. rostochiensis* and *Rhadinaphelenchus cocophilus*, the red ring nematode of coconut. Domestic quarantine regulations have also been imposed to restrict the movement of potato to prevent the spread of potato cyst nematode from Tamil Nadu to other states in India.

7. Conclusion

The plant parasitic nematodes are undoubtedly the most widespread and insidious pests of crops. The management practices against these hidden enemies of agriculture to be adopted depend upon the degree of infection, relative value of the crop, field size, level of capital investment, practicability and feasibility of the control strategy. The cultural practices are simple and effective methods of nematode pest control, adopted by the farmers. Physical methods are also simple and popular for management of nematode infestation. The biological and botanical methods are eco-friendly rather than others. Though chemical methods may create health hazards and causes environmental pollution but for urgent need and to check severe attack by serious nematode pests, and when other control measures are not

so fruitful, the chemical methods may be adopted against these noxious pests of various crops. For sustainable development of agriculture, a combination of different management systems integrated in the correct manner can help to manage the nematode problems.

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Designing Greener Pharmaceuticals and Practicing Green Health Is Required for Sustainability

Sridevi Chigurupati^{1,*}, Jahidul Islam Mohammad², Kesavanarayanan Krishnan Selvarajan³, Saraswati Simansalam⁴, Shantini Vijayabalan¹, Subhash Janardhan Bhore⁵

¹Department of Pharmaceutical Chemistry, Faculty of Pharmacy, AIMST University, Semeling, 08100, Bedong, Kedah, Malaysia; ²Department of Pharmacology, Faculty of Medicine, CUCMS, Cyberjaya, Selangor, 63000, Malaysia; ³Department of Pharmacology and Toxicology, College of Pharmacy, University of Hail, Hail, Kingdom of Saudi Arabia; ⁴Department of Clinical Pharmacy, Faculty of Pharmacy, AIMST University, Semeling, 08100, Bedong, Kedah, Malaysia; ⁵Department of Biotechnology, Faculty of Applied Sciences, AIMST University, Semeling, 08100, Bedong, Kedah, Malaysia; *Correspondence: sridevi_ch@aimst.edu.my; Tel: +6-04-429-8000 extn., 1284

Abstract: The global demand for drugs is increasing day-to-day and in the same fashion pharmaceutical effluents released from industry adversely affect the environment and human health. Discharge of pharmaceutical effluents either directly or indirectly into the environment results in substantial pollution. Disposal of such toxic effluents into the environment also affects the ecosystems either by direct or indirect pathway. Several researchers from environmental biotechnology domain have reported the presence of pharmaceuticals in water and soil. To minimize the environmental pollution and to develop the sustainable solutions, various methods to convert pharmaceutical effluents into non-toxic and biodegradable organic matter has been proposed and discussed by the scientific community. This chapter discusses the fate of pharmaceutical waste in the environment, its impact on human health, methods adopted to treat effluents before its disposal into the environment, QSAR studies in the design of biodegradable agents and future research directions to protect the environment for sustainability.

Keywords: Bioaccumulation; biodegradation; environment; green pharmaceuticals; pharmaceutical effluents

1. Introduction

In recent years, improper disposal of chemical waste has greatly affected both environment and human health. Disposal of chemical waste products as such into the environment can accumulate and affect both the biotic and abiotic factors of ecosystems. Hence, the fate of chemicals or pharmaceuticals after its disposal into the environment should be considered by the health care industry and regulatory agencies. Ecotoxicological studies of chemicals, drugs, chemical intermedi-

ates, drug metabolites and pharmaceutical waste should be performed by the manufacturer in order to know their fate in the environment. Toxic concentrations of these chemical wastes get accumulated in seawater, wastewater, sewage sludge's, water bodies and in the living organisms that are part of affected ecosystems. Environmental pollution caused by humans can adversely affect the wellbeing of both living and non-living things of ecosystem. The health of both animals and humans are affected when food materials contaminated with the toxic substances are con-

sumed. Constant pollution of the environment has disturbed several species as the toxic effluents can either destroy them or affect their reproductive life cycle permanently (Goudie, 2006; Chigurupati *et.al.*, 2016).

Recently, the concerns about environmental pollution on endocrine-related disease and disorders have been identified. Some environmental pollutants (natural and anthropogenic) interfere with endocrine system and their hormones, which can affect both animal and human health. These chemicals are called as endocrine disruptors. Proper disposal methods of chemical wastes should be considered to minimize their adverse effects on environment. To minimize the risk of environmental pollution, biodegradation is the method adopted by several chemicals and pharmaceutical industries before a chemical or pharmaceutical waste has been released into the environment. Biodegradation is a process whereby the complex organic compounds are deteriorated aerobically by microbes into simple organic matter, for instance CO₂, H₂O, NH₃, and CH₄ etc. (OECD Statistics Directorate, 2002).

This chapter highlights the fate and impact of pharmaceutical waste in the environment and on human health, respectively. Various methods used to treat effluents, the role of QSAR studies in biodegradation and the possible research directions to protect the environment from toxic effluents are also discussed.

2. Pharmaceutical biowaste and its environmental impact

In general, man-made biowaste causes the environmental pollution and can pose a threat to the public health. Biowaste originates from various sources as stated below (Rand-Weaver *et al.*, 2013):

- Formulation and industrial set up.
- Management and packing of hazardous chemicals along with go-

downs, warehouses or tanks used in fuel and chemical industries.

- Carriage i.e. air, road, rail, pipelines, and water.
- Impurities emission such as Nitrogen dioxide, Carbon monoxide, particulate matter ($\leq 10\mu\text{m}$), total suspended particulate matter, Sulphur dioxide and volatile organic compounds such as acetonitrile, dichloromethane, ethylene glycol, methanol, N,N dimethyl formamide and toluene.
- Various types of effluents (may be lethal in nature) those are not effectively biodegraded. The effluents those are able to go straight towards oceans, lakes, rivers, streams or other bodies of water. The discharge because of overflow, including storm water run-offs, could likewise be a potential risk.

Figure 1 depicts that how drug metabolites or drugs reaches water bodies and results in contamination of environment. In addition to health benefits, pharmaceuticals (drugs, chemicals, pills, medicine, sedatives, stimulants, narcotics etc.) are also known to cause various types of damage and or pollution. The risks from the pharmaceuticals could be categorized as (Calleja *et al.*, 1994):

- Ecotoxicological damage elicited to the environment.
- Malignant- contribute to the causality of cancer.
- Persistent-remain hazardous for a long time.
- Bio-accumulative-gathers as it make its way up the food chain.
- Disastrous due to a catastrophe, mishap, calamity or grave manifestation in any area

Seepage of the chemicals or pharmaceuticals to the environment is common from the usage of diagnostics, antiseptics and individual care products. The pharmaceuticals, their metabolites

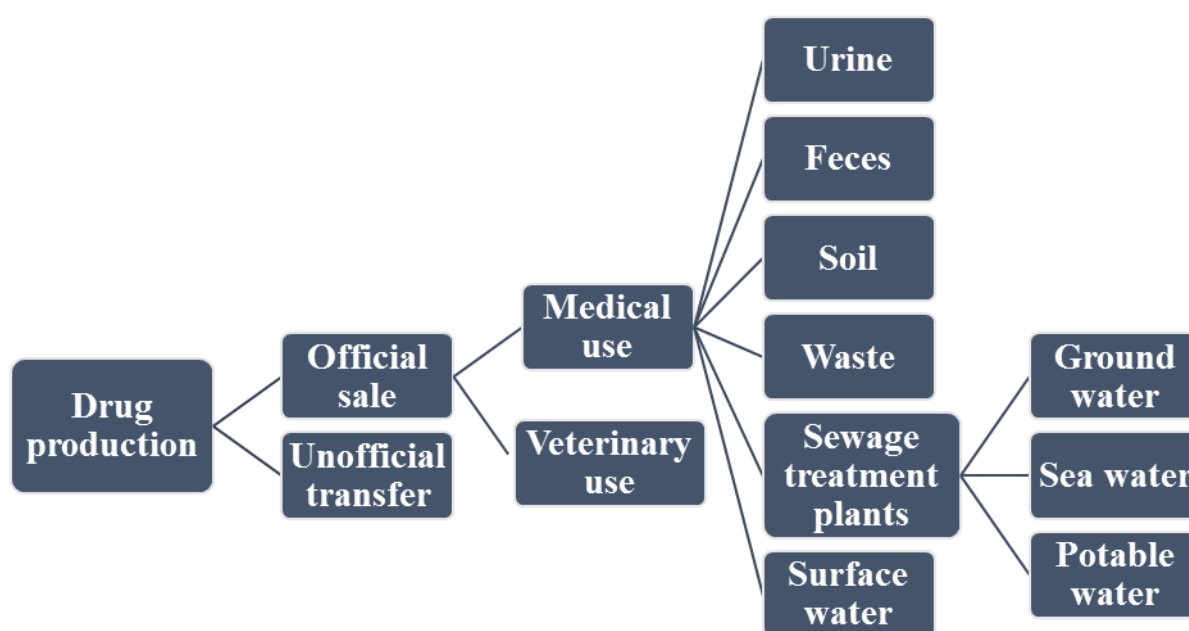


Figure 1: A diagrammatic sketch showing imaginable sources and paths for the pharmaceutical deposits to reach into the water bodies including our drinking water.

and transformation products if not eradicated during sewage handling then they may come in the aquatic environment and in the long run can reach the drinking water bodies and may come to our homes through water supply (see Figure 1). The active amalgams pharmaceuticals or its derivatives could enter into environment by various routes or non-point sources; for instance waste, sewage treatment plants (STPs) and landfill effluent or from animals treatment facilities (Calamari *et al.*, 2003).

Pharmaceuticals, particularly hormones were the primary focus of research and public consciousness in the 1970s. The hormones do not (bio) degrade completely. However, the concept produced enthusiasm in 1980s. Other constituents like heavy metals, aromatic polycyclic hydrocarbons, pesticides and detergents were also the issue of widespread examination during 1980s (Jones *et al.*, 2008).

Deliberate research on contamination of environment by various pharmaceuticals is undertaken by some research

teams in few countries. However, the presence of approximately 160 various drugs and or various pharmaceuticals chemical associates is reported in STPs effluent and in ground and surface water bodies (WHO, 2011). Even in drinking water samples, some Active Pharmaceutical Ingredients (APIs) were found (WHO, 2011). They are additionally distinguished in the freezing environment. Contrasted with the free water, phase analysis of APIs is difficult in bio solids and sewage sludge for proper comprehension (Daughton and Ternes, 1999).

Medications are useful in aquaculture, livestock farming, and veterinary and in other sectors of agriculture. However, improper practices results in contamination of environment; for instance, seepage of growth promoters are found in contaminated soil. Various types of chemicals used in agriculture and those become source of environmental contamination or biowaste can have an adverse impact on human health (Table 1). Veterinary antibiotics could end up in the groundwater or in the soil. By runoff, it may be cleared

Table 1: Different types of wastes and their impact on human health (Ferrari *et al.*, 2003; Tauxe-Wuersch *et al.*, 2005; Isidori *et al.*, 2006; Khanal *et al.*, 2006)

No	Type of waste	Effect on humans
1.	Nuclear waste	Hair: The losing of hair occurs with radiation exposure at 200 rems. Brain: Brain cells do not replicate, they damaged directly at the exposure is 5,000 rems. Thyroid: The thyroid gland is vulnerable to radioactive iodine. In adequate amounts, radioactive iodine can destroy all or part of the thyroid. Blood System: The blood's lymphocyte cell count will be reduced once a person is exposed to around 100 rems, leaving the victim more susceptible to infection. This is often referred to as mild radiation sickness. Heart: Immediate damage to small blood vessels and perhaps cause heart failure and death directly by intense exposure to radioactive material at 1,000 to 5,000 rems. Gastrointestinal Tract: Exposure to 200 rems or more radiation harm to the intestinal tract lining will cause nausea, bloody vomiting and diarrhea. The radiation will begin to destroy the cells in the body that goes division rapidly. These including blood, GI tract, reproductive and hair cells, and harms their DNA and RNA of living cells. Reproductive Tract: Since reproductive tract cells multiply rapidly, these areas of the body can be damaged at rem levels as low as 200. Long-term, some radiation sickness victims will become sterile.
2.	Environmental waste	Interaction of humans to agrichemicals is common and results in acute and chronic health threats, together with acute and chronic neurotoxicity (fumigants, insecticides and fungicides), lung chemical burns (anhydrous ammonia), newborn methemoglobinemia and also impairment (paraquat).
3.	Agrichemicals	Soil pollution effects causes leukemia and it is danger for young children as it can cause developmental damage to the brain furthermore it illustrated that mercury in soil increases the risk of neuromuscular blockage, causes headaches, kidney failure, depression of the central nervous system, eye irritation and skin rash, nausea and fatigue. Soil contamination closely related to air and water pollution, so numerous things come out as similar as caused by water and air pollution.

into surface water bodies. Wide variety of indications of various active ingredients in liquid state and in the soil has been established. The primary reviews that have researched the transfer and the related risks have been published only recently (Corcoran *et al.*, 2015).

Antibiotics transpire naturally into the soils and the resistance against these antibiotics influences the inhabitant's dy-

namics in soils. The profusion of these natural antibiotics was less and appeared as restricted to the adjacent surroundings (Kümmerer, 2004). The composition of the soil-lodging species is found to be influenced by antimicrobial agents. Antibiotics in the soil appear to support fungal development. The circumstances in the areas beneath fish farms are critical due to more concentrations of antimicrobial

agents as a result of seepage. Some antimicrobial agents are responsible to diminish the number of microscopic organisms around aquaculture facilities or where the seepage of antimicrobial agents was noticeable (Oliveira *et al.*, 1995). The degree of the impact of ciprofloxacin on microbial salt marsh groups was contrarily related to the level of sorption to the sediments. Even though ciprofloxacin is a wide-spectrum antibiotic, its influence on sediment microbial organisms was selective and seemed to favor Gram-negative and sulfate-reducing bacteria. (Sengupta *et al.*, 2013). This type of the environmental contamination can cause the imbalance in ecosystems.

The impacts of oxytetracycline in environmentally applicable concentrations on enchytraeids, springtails and earthworms have been analyzed. Neither one of the antibiotics demonstrated any harmfulness against the organisms under scrutiny. However, the capability of a pollutant to accumulate in organisms must be considered seriously. Antibiotics that are inadequately water soluble, particularly if the bio-concentration factor is between 500 and 1000 or if the octanol/water distribution coefficient crosses the value of 1000, have a tendency to get accumulated in organisms. The substance enrichment in organisms has been proved for a few antibiotics, e.g., sulphadimethoxine (Call *et al.*, 2013).

3. Biodegradation of pharmaceutical products

Globally, the production of pharmaceuticals continues to grow year by year, and with its environmental concerns pertaining to not just production, but also consumer waste and disposal (Wu *et al.*, 2010). The drugs (pharmaceuticals) consumed are eventually either excreted by humans and animals and or disposed which causes environment pollution. Most of the pharmaceuticals produced, finally make their way to the ecosystems and causes environmental pollution as

well as affects the flora and fauna at various extents. Hence, the dumping of pharmaceuticals into the environment is now recognized as a serious issue (Boxall, 2004).

There are many techniques and or processes utilized to overcome this type of pollution problem. Few of them are - (i) Thermal process; (ii) Chemical process; (iii) Irradiation process; (iv) Biological process (biodegradation); and (v) Mechanical process.

Among the five above stated processes used to minimize the waste or pollution, biodegradation techniques (biological process) are considered as greener technique/technology that involves the use of various biological processes to overcome the adverse effects of pharmaceutical effluents into the environment. Biodegradation is a complex and natural decomposition process of organic substances with the help of biochemical reactions of microbes (OECD Statistics Directorate, 2002). Although, pharmaceuticals signify a minor fraction of all chemicals that are dumped into the environment, exceptional care must be taken about their presence in the environment. Because, they are ubiquitous and disseminate easily; they act on biological systems; they show a lot of side effects in non-targeted bodies; and they are known to cause chronic toxicity even at low concentrations (Enick and Moore, 2007).

As stated by Jones *et al.* (2005), the occurrence of pharmaceutical contaminants in the environment was reported first time in late 1970's (Jones *et al.*, 2005). The major source of pharmaceuticals in the environment is due to their improper disposal and leaching from landfill sites to natural water (Jones *et al.*, 2004) and most often the drugs enter into sewages and culminate in water sources. There is no any guarantee that the pharmaceutical drugs and their effluents are removed by merely dumping in sewage (Boxall, 2004). Moreover, many times, it is proven that the water waste treatment

plants are inefficient in managing the pharmaceutical effluents (Ternes, 1998).

The main biodegradation techniques are - (i) aerobic biodegradation; (ii) anaerobic biodegradation, and (iii) biodegradation in activated sludge and pure culture.

3.1. Biodegradation by Aerobes

Aerobic biodegradation is the breakdown of organic substances by microorganisms in the presences of oxygen. The microorganisms used in the process require oxygen for growth; hence these microbes are called as aerobes. Aerobic degradation of hydrocarbons is an example of this biological process. Aromatic hydrocarbons could be converted into natural intermediates; for instance, protocatechuate and catechol (Figure 2) (Boxall, 2004). Gram negative bacteria that possess plasmids produce enzymes needed for the aromatic compounds degradation. This is possible because of hydroxylation reaction which introduces a hydroxyl group (-OH) into substrate, in this case hydrocarbons (Chatterji, 2003).

3.2. Biodegradation by anaerobes

Anaerobic biodegradation is the breakdown of organic substances by using microbes in the absences of oxygen. When the anaerobes are predominant over the aerobic microbes then anaerobic biodegradation is preferred. It is broadly used to treat biodegradable waste and wastewater sludge since it gives mass and volume reduction. There are four key biological and chemical reactions involved in anaerobic biodegradation namely, hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Figure 3).

3.3. Biodegradation in activated sludge and pure culture

Activated sludge treatment is the most well-known process for the wastewater treatment. Two disposal forms act simultaneously in the activated sludge, either the biodegradation by the microbes or sorption to solids.

Example: Compounds like mefenamic acid and ibuprofen, the greater part of the expulsion is because of biodegradation (Jones *et al.*, 2007). The simpler a molecule, the higher chance for biodegradation to occur (Jones *et al.*, 2004). During biodegradation, the xenobiotic compounds can be changed in three diverse ways namely, hydrophobic, hydrophilic and or mineralization transformation. Carbamazepine (CBZ) is one of the pharmaceuticals with the lowest removal efficiency when treated with activated sludge treatment (Ternes, 1998). Subsequently, CBZ is not influenced either by sorption or by the microbes. Most positive outcomes with respect to the biodegradation of CBZ are by a pure culture.

4. QSAR studies in biodegradation

Persistent, bioaccumulative, and toxic chemicals show high lipid solubility and low water solubility which lead to high potential for bioaccumulation. The European REACH regulation is the first to work on quantitative structure–activity relationship (QSAR) models for biodegradation. REACH inspires the use of substitutes to animal testing which includes predictions from QSAR models. QSAR Model mainly involves three types of data generation - Persistence data generation; biodegradation data generation; and toxicity data generation. For estimating biodegradation, there are few important data bases such as Syracuse BIODEG, BIODEG database, BIOLOG Database, MITI Database, and ESIS Database.

The partially observed persistence; bioaccumulation and toxicity data, the expensive testing composed with the regulatory limitations and the international encouragement to minimize animal models motivates a greater dependence on QSAR models in the biodegradation assessment. Several QSAR biodegradation models have been established for selected groups of structurally similar compounds like: specific number of alcohols, chlorophenols, n-alkyl phthalates, chloroanisoles,

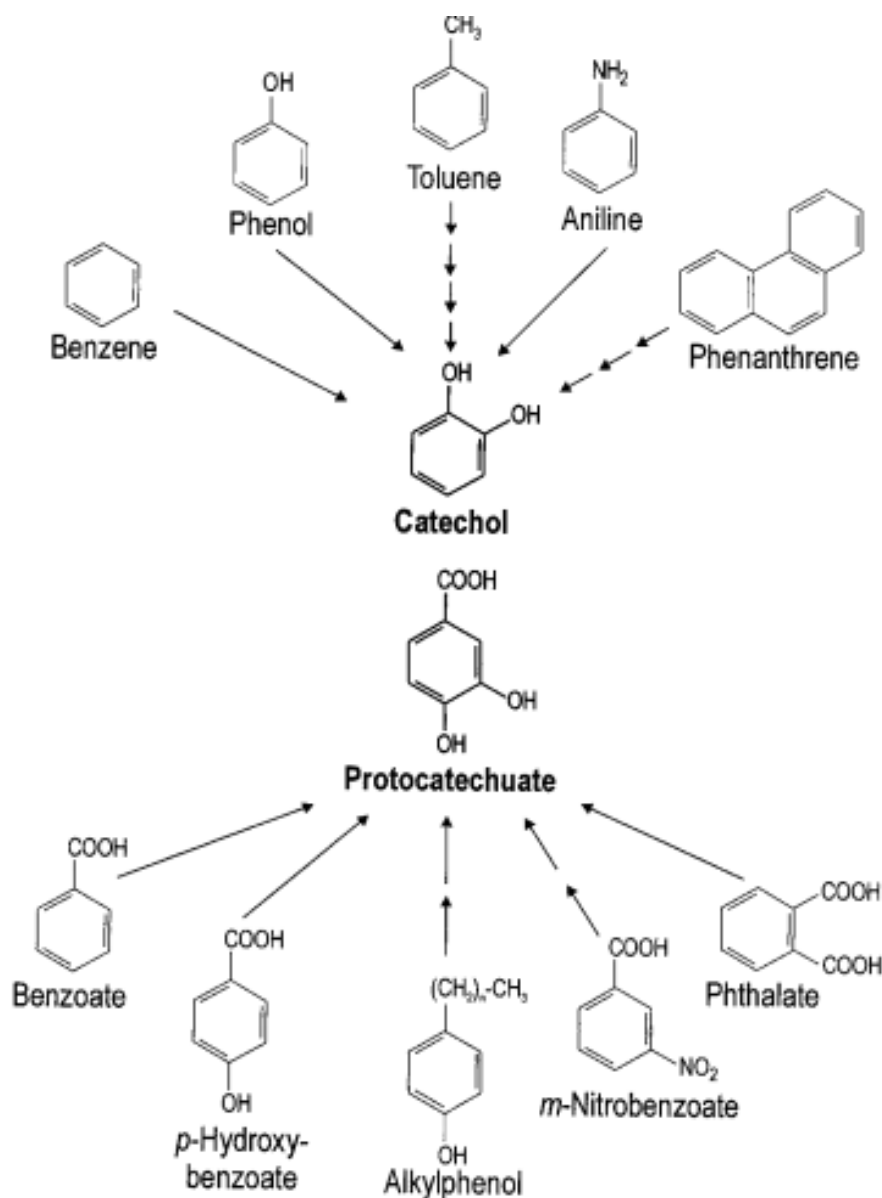


Figure 2: A diagrammatic sketch showing degradation of natural aromatic and some xenobiotic compounds.

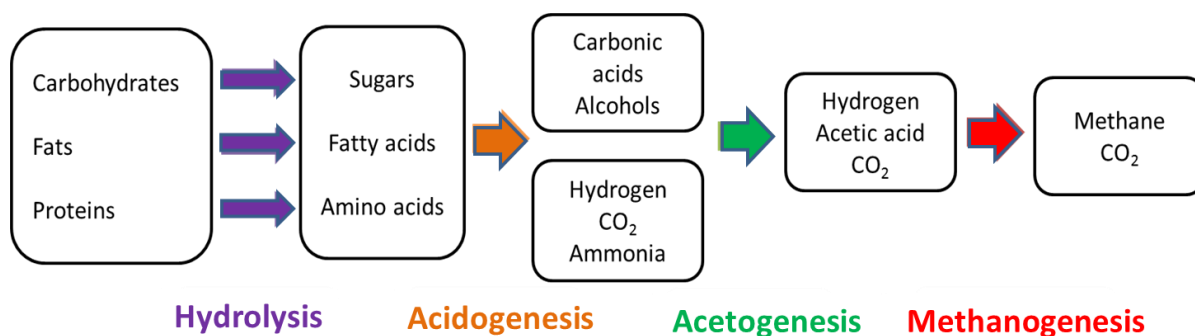


Figure 3: Schematic diagram showing anaerobic biodegradation of xenobiotics.

meta-substituted anilines and para-substituted phenols etc. Most of the Quantitative structure- biodegradation relationships (QSBRs) depend on the octanol/water partition coefficients, alkaline hydrolysis rate constants, van der Waals radii and also on molecular connectivity indices. Generally, the association between physicochemical properties or molecular descriptors and biodegradation rates were satisfactory; but, generally these models have not been used much. Because, use of these models is limited to the specific classes of chemicals for which these models were created. Hence, these models are unsuitable to predict biodegradation rates for chemicals outside of those classes (Howard *et al.*, 1992; Dimitrov *et al.*, 2005).

Therefore, in recent years a very rigorous development of new and better qualitative and quantitative biodegradability models by the usage of new and well developed statistical and computational methods are developed. Weighted molecular fragments are used as model descriptors with an idea that molecular fragments may have an attractive or hindering effect on biodegradability. Numerous statistical techniques have been used in determining weights: linear and non-linear regression modelling partial least square (PLS) and neural networks. The results of QSBR studies are strongly influenced by the way the molecule is fragmented. To overcome this, the Multi-CASE approach has been established to generate all possible fragments of the molecules and to subsequently select the statistically most significant ones to suit proper results. These fragments are then used to develop regression models between screened fragments and the final results (Banerjee *et al.*, 1984; Niemi *et al.*, 1987).

5. Test guidelines for degradation and bioaccumulation studies

Biodegradability test is performed to evaluate the fate of a chemical sub-

stance in the environment. The information obtained from the degradability test is mainly required for its hazard or risk assessment in aquatic environment. The waste water get in contact with the sewage treatment microbes and chemical substances enter into the oceanic environment. The process whereby the chemical substance gets bioaccumulated in an (aquatic) organism is known as bioaccumulation

The bioaccumulation degree of a chemical substance at a given period is the collective data of the competing processes of uptake, distribution, transformation and excretion. The information on degradation and accumulation could be obtained by performing appropriate standardized tests recommended by the OECD (Table 2).

6. Malaysian government regulations on pharmaceutical pollution

In 2005, the Ministry of Natural Resources and Environment (MNRE) has published guidelines on the disposal of clinical and pharmaceutical wastes. Any waste listed in the First Schedule of the Environmental Quality (Scheduled Waste) Regulations known as Scheduled Waste, this type of waste can only be disposed in the predetermined premises and waste must be treated before its disposal. These wastes may possess either inorganic or organic components, e.g. discarded drugs comprising psychotropic or dangerous substances such as carcinogens, mutagens or teratogens (SW 403) (Ministry of Natural Resources and Environment, 2009).

In 2010, the Pharmaceutical Services Division initiated 'Return Your Medicines Programme' in which patients were encouraged to return the expired and unused drugs to the pharmacies in the government hospitals. The aim of this programme was to promote safe disposal of medications and mitigate the undesired adverse effects of APIs on the environment as well as living beings. At the end

Table 2: OECD test guidelines to assess the environmental fate of chemicals (OECD Guidelines for the testing of chemicals - OECD, no date)

Test No	Test Title
301	Ready Biodegradability
302A	Inherent Biodegradability: Modified SCAS Test
302B	Inherent Biodegradability: Zahn-Wellens/ EVPA Test
302C	Inherent Biodegradability: Modified MITI Test (II)
303	Simulation Test - Aerobic Sewage Treatment - A: Activated Sludge Units; B: Biofilms
304A	Inherent Biodegradability in Soil
305	Bioaccumulation in Fish: Aqueous and Dietary Exposure Bioconcentration: Flow-through Fish Test
306	Biodegradability in Seawater
307	Aerobic and Anaerobic Transformation in Soil
308	Aerobic and Anaerobic Transformation in Aquatic Sediment Systems
309	Aerobic Mineralization in Surface Water – Simulation Biodegradation Test
310	Ready Biodegradability - CO ₂ in sealed vessels (Headspace Test)
311	Anaerobic Biodegradability of Organic Compounds in Digested Sludge: by Measurement of Gas Production
312	Leaching in Soil Columns
313	Estimation of Emissions from Preservative - Treated Wood to the Environment
314	Simulation Tests to Assess the Biodegradability of Chemicals Discharged in Wastewater
315	Bioaccumulation in Sediment-dwelling Benthic Oligochaetes
316	Phototransformation of Chemicals in Water – Direct Photolysis
317	Bioaccumulation in Terrestrial Oligochaetes

of year 2016, the Ministry of Health (MoH) had disposed nearly RM 2 million worth of medicines, most of which were obtained through the 'Return Your Medicines Programme'. The most common medicines returned under this programme were the ones used for treatment of diabetes, hypertension, hyperlipidemia and gastritis. The common reason for the patients to incomplete the course of medicines was a change or discontinuation of a treatment (*Ministry destroys RM 2 million worth of expired meds - Nation | The Star Online*, 2016).

7. Concluding remarks

The pharmaceuticals should be designed by using green chemistry techniques; so that at the end, products can be easily broken down into innocuous degradation products and do not persist for

long time in the environment. The organic carcinogenic solvents should be replaced with green solvents wherever it is possible. Industries should more focus on the minimum utilization of atoms for the synthesis of pharmaceutical products.

Biological based products are naturally less poisonous and promote the principles of green chemistry by expanding prospects to develop expected processes exploiting renewable resources. Ultimately, renewable resources do have a potential to produce a significant amount of pharmaceutical materials which are currently produced using either hazardous and or nonrenewable materials. Green chemical synthetic procedures should replace the conventional procedures of producing pharmaceuticals. It is not only essential to promote the health of people but also for the sustainability of the industry and planet.

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Clonal Propagation of a High Value Multipurpose Timberline Tree Species *Quercus semecarpifolia* Sm. of West Himalaya, India

Aseesh Pandey^{1,2} and Sushma Tamta^{2,*}

¹Govind Ballabh Pant National Institute of Himalayan Environment and Sustainable Development, Sikkim Unit Pangthang 737101, East Sikkim, Gangtok, India; ²Plant Tissue Culture Laboratory, Department of Botany, DSB Campus, Kumaun University, Nainital 263001, Uttarakhand, India; *Correspondence: sushmatamta@gmail.com; Tel: +918126966284

Abstract: Tree species across the alpine timberline are most vulnerable to climate change and requires immediate attention for their conservation. *Quercus semecarpifolia* Sm. forms the extensive forests in alpine timberline region and it is among the highly exploited tree species of western Himalaya. Attempts were made to develop *in vitro* clonal propagation procedure for *Q. semecarpifolia*. Nodal explants, derived from a single mature tree growing in natural stands, were cultured on different nutrient media for the optimization of nutrient medium. Woody plant (WP) medium supplemented with 8.88 μ M 6-benzylaminopurine (BA) and 0.72 μ M Gibberellic acid (GA_3) has yield significant shoot multiplication response. For root induction a two- step method was applied. Microshoots treated with 100 μ M indole-3-butyric acid (IBA) for 24h, showed the significantly higher rooting response. Present study leads a way forward to conservation and sustainable utilization of this high value tree species. Moreover, further strengthening and optimization efforts are required to develop a low cost procedure for the development of the nursery of clonally propagated *Q. semecarpifolia* plants. Thus, the threat-mitigation strategies can be developed through the introduction of these *in vitro* raised plants into the natural forest.

Keywords: Clonal propagation; nodal explant; *Quercus semecarpifolia*; timberline

1. Introduction

Alpine timberline is the most sensitive ecotone to changing climate (Smith *et al.*, 2009). The regeneration of tree species in this region is expected to affect adversely in coming years. Ample evidences of a typical regeneration of timberline tree species are already documented by various researchers across timberlines of different mountains located in diverse geographical provinces (Harsch *et al.*, 2009; Walck *et al.*, 2011; Kirdeyanov *et al.*, 2012). The global mean surface temperature has risen by 0.6°C over the last 100 years (Wang *et al.*, 2016), and the level of precipitation in Northern Hemi-

sphere has increased by approximately 5-10% over mid and high latitudes (IPCC, 2013). Due to diverse climatic conditions, topography, and precipitation regimes the composition of timberline varied across the globe and dominated by different tree species. The Himalaya harbors a unique mountain system and represents the highest alpine timberline of the world. The timberline of Indian Himalayan region (IHR) is dominated by different tree species from east to west. The IHR consists of more than 35 species of genus *Quercus* (Singh and Singh 1992). The genus *Quercus* is represented by five evergreen tree species (*Q. leucotrichophora*, *Q. floribunda*, *Q. glauca*, *Q. lanuginosa* and *Q.*

semecarpifolia) and one deciduous exotic tree species (*Q. serrata*) in Uttarakhand, Western Himalaya, India (Pandey and Tamta, 2012). Among these, *Q. semecarpifolia* (Kharsu) possess highest elevational range (2500-3300m asl) and forms timberline in the region (Singh and Singh, 1992).

The world's highest timberline is also utilized by local peoples for their daily needs (agricultural tools, fuel, food, fodder, spiritual rituals etc.) and livelihoods (agriculture, silk worm rearing, livestock rearing, etc.). This dependency creates enormous pressure on multipurpose evergreen tree species like *Q. semecarpifolia*. This species is considered as the oldest and overexploited plant of sub-alpine zones (Singh *et al.*, 2010). The green leaves of *Q. semecarpifolia* are used in tasar silk-worm rearing and livestock fodder; bark and galls for tannin; dried branches and boles are used in the preparation of agricultural implements and used as fuel wood (Tamta *et al.*, 2008; Pandey, 2013). This high magnitude of human pressure along with low regeneration (Bisht *et al.*, 2012), short viability of seeds (Pandey and Tamta, 2013), and unavailability of a decent seed crop every year (Pandey 2013) could be precarious for the natural regeneration of this species in changing climate. Considering the role of *Q. semecarpifolia* in the economy as well as ecology of Himalaya, immediate attention is required for the conservation of this species through sustainable utilization of its genetic resources. The *ex situ* conservation through plant tissue culture technique is extensively used method for the conservation and sustainable harnessing of use values of threatened and high value woody species such as *Citrus sinensis* (Pandey and Tamta, 2016); *Berberis aristata* (Brijwal *et al.*, 2015); *Quercus serrata* (Pandey and Tamta, 2014); *Berberis chitria* (Pandey *et al.*, 2013); *Quercus semecarpifolia* (Tamta *et al.*, 2008) and other *Quercus* species (Wilhelm, 2000). Based on the source of explant, the plant tissue culture technique

is comprises of various propagation methods. However, the clonal propagation through nodal explants derived from mature trees growing in natural forest is tedious, still one of the best method to develop true-to-type plants of desired quality. In present study attempts were made to develop clonal propagation procedure for *Q. semecarpifolia* using nodal explant derived from mature elite tree growing in *Q. semecarpifolia* dominated forest, west Himalaya India.

2. Material and methods

2.1. Plant material

Nodal explants were collected from the mature tree growing in China peak forest area (2619m asl; 29° 27'N and 79° 29'E) of district Nainital, Uttarakhand, India. Twigs (10-15 cm) were collected from different parts of mature trees (from stump sprouts, or from juvenile parts) and brought to the Plant Tissue Culture laboratory, Department of Botany, D.S.B. campus Kumaun University, Nainital, India in a mini-chillier (-10°C; Genei, Bangalore). In laboratory, the twigs were dipped in water and kept in room temperature for 1h. Within same day, the twigs were defoliated and cut in to small segments (1.0-1.5cm). These nodal segments having at least one dormant bud were used as explant.

2.2. Chemicals, glassware and culture conditions

Each chemical used during the experiment was of analytical grade and purchased from Himedia, Pvt. Ltd. Mumbai, India except labolene (Qualizen, India) and Bavistin (purchased from local market). The plant growth regulators were procured from Duchefa Biochemie, The Netherlands. All the glasswares used during the experiment were purchased from Borosil India. Cultures were maintained at 25±1°C under a 16h/8h light/dark photoperiod with an irradiance of 42 $\mu\text{mol m}^{-2} \text{s}^{-1}$ inside and 60 $\mu\text{mol m}^{-2} \text{s}^{-1}$ outside the culture vessels/ flasks pro-

vided by cool fluorescent tubes (40 W; Philips from Saveer Biotech Limited, New Delhi, India).

2.3. Explant preparation

Nodal explant were initially washed in running tap water for 10 min and then immersed in detergent solution (labolene, 0.1%, v/v; 20 min), and rinsed thoroughly with distilled water. Washed explants were treated with fungicide (bavistin, 0.5%, w/v; 30 min) with gentle shaking. Those treated explants were introduced to laminar air flow cabinet, where after 5 rinses of autoclaved double distilled water, nodal segments were surface disinfected with mercuric chloride (HgCl_2 , 0.1%, w/v; 10 min). This treatment was followed by subsequent 5 rinses of autoclaved double distilled water (2 min each). The exposed ends of nodal segments were excised out prior to inoculation. To check the contamination nodal explants were cultured in WA: water agar medium for one week (Figure 1a).

2.4. Shoot induction and nutrient medium selection

To select the nutrient medium for shoot induction and multiplication, contamination-free nodal segments were inoculated either in full strength MS: Murashige and Skoog (1962) or WP: Lloyd and McCown (1980) nutrient medium. Each medium was supplemented with 6-benzyleadenopurene (BA, 4.44 μM) and activated charcoal (AC, w/v, 20mg l^{-1}) to monitor the shoot induction responses.

2.5. Shoot multiplication

After 30 day on shoot induction media, explants with positive shoot induction response were transferred to shoot multiplication media (Figure 1). Different concentrations of cytokinin (BA, 4.44–22.20 μM) alone or in combination with IAA (1.43 μM) or GA_3 (0.73–1.44 μM) were tested for shoot multiplication responses in WP full strength nutrient medium. The sucrose concentration was 3.0%

(w/v) and the media was solidified with 0.8% agar (w/v) and/or 0.24% clarigel (w/v). Besides, casein hydrolysate (CH, w/v, 500mg l^{-1}) and activated charcoal (AC, w/v, 20mg l^{-1}) were also incorporated in to each shoot multiplication medium. Each experiment consisted of 12 explants and repeated twice. Subculturing was carried out at 6 week interval and data on shoot number and shoot length were recorded during subculture.

Explant selection experiment was carried out in culture vessels (50 ml volume, 20 ml medium per tube). The contamination-free explants were transferred to conical flasks (250 ml volume, 100 ml medium per flask) for further growth and development. WP basal media devoid of growth regulators served as control in all experiments. All culture vessels and flasks were plugged with non-absorbent cotton plugs and sterilized at 121°C for 15 min. The pH was adjusted to 5.7–5.8 by 0.1M NaOH or HCl before autoclaving at 1.06 kg cm^{-2} (121°C) for 20 min.

2.6. Rooting of microshoots

Microshoots (2.0–3.0 cm, with well-developed leaves) were introduced to a two step-rooting procedure; as described by Pandey and Tamta et al (2012). Briefly, during the first step excised microshoots were cultured in full strength WP media supplemented with different concentrations of indole-3-butyric acid (IBA 50.0 or 100.0 μM) for 24 or 48 h and cultures were placed in a dark during this step (Table 2). In the second step these treated shoots were transferred to PGR-free half-strength WP medium, solidified with clarigel (0.25%) and exposed to 16 h photoperiod. The percentage of root formation, lengths of the formed roots and length of longest root were recorded after 4 weeks of incubation in PGR-free half-strength WP medium.

2.7. Acclimatization

After 4 week of transfer to PGR-free medium, these shoots with roots were taken out from the culture flasks and wa-

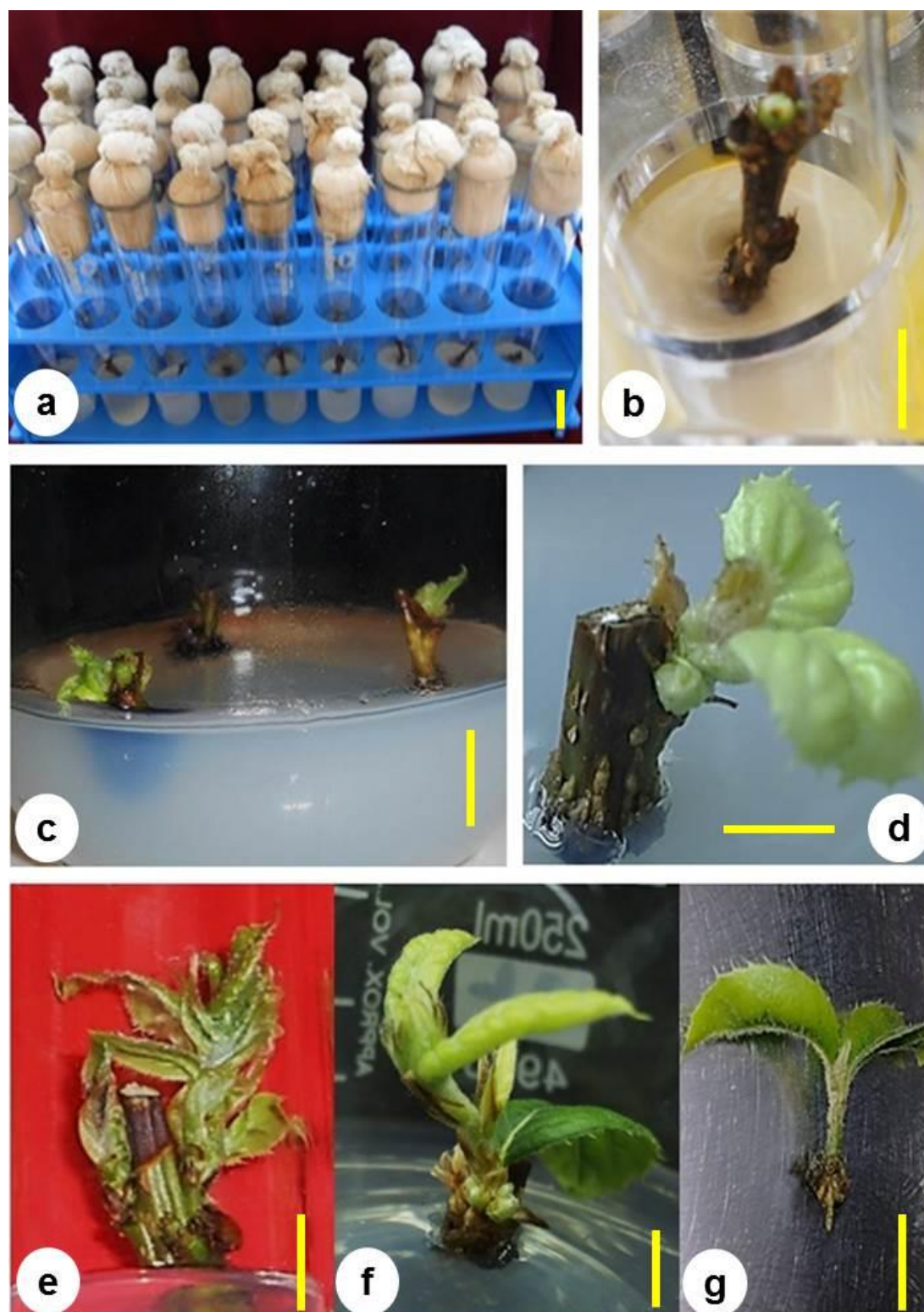


Figure 1: *In vitro* propagation of *Quercus semecarpifolia*. **a)** Explant inoculation in WA medium to check contamination; **b)** contamination free explants having positive response; **c-d)** Shoot proliferation in WP supplemented with (BA 4.44 μ M); **e)** shoot elongation in BA+GA₃ (4.44 μ M +1.45 μ M); **f)** shoot multiplication in BA+GA₃ (8.88 μ M +1.45 μ M); **g)** root induction in IBA 100 μ M for 24 h.

-shed gently with distilled water to remove traces of clarigel. After recording of data, plantlets were transferred to therma-

col pots (8 cm width and 10 cm height) containing soil and farmyard manure (3:1, w/w). These plants were placed inside

growth chamber under 16 h photoperiod ($60\mu\text{mol m}^{-2}\text{s}^{-1}$) at $25\pm 2^\circ\text{C}$ temperatures and 60% relative humidity. Plants were watered on alternate days with 1/4 basal WP medium devoid of sucrose and acclimatized over a period of 4 week.

2.8. Statistical analysis

Experiments were performed in a completely randomized design to determine the effect of treatments and concentrations on plant vigour. Data presented as mean values $\pm$ standard error (SE) and collected from three independent experiments. The statistical analyses were performed using SPSS (Statistical Package for Social Science, version 20). Level of significance was determined by analysis of variance (ANOVA) and statistical significance mean values were grouped by using Duncan's multiple range post hoc test ($p < 0.05$).

3. Results

3.1. In vitro culture establishment

Nodal explants cultured on WA medium had shown high contamination and poor survival (10-15%) rate. The survival rate could not be maximized even after altering the concentrations and exposure time of disinfectants (data not shown). Further, the high phenolic secretion from basal end of nodal segment was also observed. Nonetheless, with the increase in concentration and exposure time of disinfectant, explants started to die. The contamination-free explants were used for shoot induction.

3.2. Shoot induction

Both tested media (MS or WP) supplemented with BA ($4.44\mu\text{M}$) were able to induce shoots (Figure 2), nevertheless, the shoot induction rate and response time was different. WP basal medium fortified with BA ($4.44\mu\text{M}$) gave better results. Average shoot induction (88.89%) was recorded within (14.00 ± 2.03 days) response time. While comparatively less percentage of shoot induction (77.78%)

was recorded in MS medium supplemented with similar concentration of BA ($4.44\mu\text{M}$) with higher response time of 17.44 ± 2.12 days. The activated charcoal particles (gas absorber) were found more suitable than activated charcoal powder. The incorporation of activated charcoal in to shoot-induction media has shown promising results (data not shown) in shoot induction and incorporated in to the shoot multiplication media.

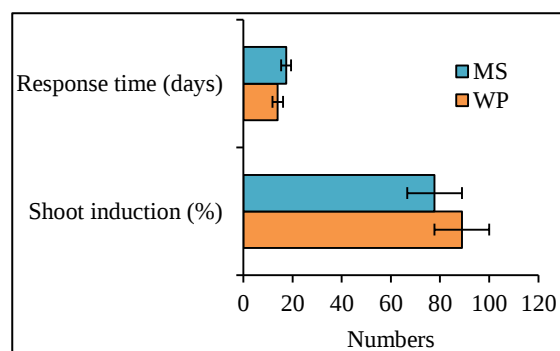


Figure 2: Effect of nutrient mediums on *in vitro* propagation of *Q. semecarpifolia*. MS, Murashige and Skoog (1962) medium; WP, woody plant medium (Lloyd and McCown, 1980).

3.3. Shoot multiplication

Explants with positive responses (induced shoots; Figure 1b-c) were sub-cultured in the shoot multiplication medium, supplemented with different concentrations of BA alone or in combination with GA_3 or IAA (Table-1). After six week of culture, the shoot multiplication rate differed significantly ($p < 0.05$) among the treatments. In alone BA supplemented media, the shoot number was significantly ($p < 0.05$) increased with the increasing BA concentrations from $4.44\mu\text{M}$ to $22.22\mu\text{M}$. The significantly ($p < 0.05$) highest number of shoots were (7.44 ± 0.44) observed in WP medium supplemented with BA+IAA ($17.76\pm 1.43\mu\text{M}$) (Plate-1d,f). Overall, the significantly ($p < 0.05$) best shoot multiplication response (5.89 ± 0.59 shoots/explant) with the average shoot length of 3.37 ± 0.29 cm and 4.02 ± 0.40 cm average length of longest shoot was observed in WP medium supplemented with

BA+GA₃ (8.88±0.72 µM) (Figure 1e). Though the number of shoots were significantly ($p < 0.05$) less in BA+GA₃ supplemented media but the other parameters were recorded significantly ($p < 0.05$) better in comparison to other used treatments. The explants cultured in control treatment had not shown any shoot multiplication response.

3.4. Rooting of microshoots

Root induction visually observed after 10 days of transfer in ½ strength WP media. Shoots having root length >1mm considered as rooted. All IBA treatments were able to induce roots in microshoots; however, the maximum rooting (88.89±1.11) was observed in the medium supplemented with 100.0 µM IBA (Figure 1g). A 3.63±0.23 number of roots were observed within 4 weeks with an average root length of 0.86±0.01cm (Table-2). Root formation was not observed in microshoots that lack IBA in the first step of root induction method. Although root induction takes place by applying two step methods but these rooted plantlets were not able to survive in transplanted soil conditions and died after 20 days.

4. Discussion

Sterilization of *explants* is the most important step of plant tissue culture. For present study two well-studied sterilizing agents (Mercuric chloride and Bavistin) were used. Bavistin is systemic fungicide generally used against the members of ascomycetes, deuteromycetes and various basidiomycetes (Vyas, 1984). Mercuric chloride (HgCl₂) used to control various microbial infestations (Bonga and Aderkas, 1992), and it is effectively used for the surface sterilization of several hard wood species (Chalupa, 1987). In present study both used sterilents were not able to control contamination rate and only 10-20% contamination-free explants were achieved. On increasing the concentration or treatment time of HgCl₂ the percent survival was further decreased; this may

because of the ability of HgCl₂ to penetrate the cutinized cell wall, which leads to precipitation of cell protein (Sharma and Sharma, 1980).

Charcoal is a form of carbon having a high adsorptive capacity of gases, vapours and colloidal solids (Pan and Staden, 1998). The activated charcoal used in WP nutrient media has an adsorption preference for moderately polar rather than a polar or highly polar organic chemicals and they show greater adsorption for aromatic than olefinic unsaturation products (Yam *et al.*, 1990). Therefore, aromatic compounds such as the phenolic compounds secreted from nodal explants and their oxidates, could have great adsorption affinity for activated charcoals. Nevertheless, the highly polar and readily water-soluble sugars (glucose, sorbitol, mannitol and inositol) might not be removed from the medium and/or solution (Pan and Staden, 1998). This property of activated charcoal might have enhanced the shoot induction rate in present study without altering the basic composition of WP nutrient medium. Activated charcoal also controlled browning and stimulated shoot growth of *Strelitzia reginae* and *Anemone oronaria* (Mensuali-Sodi, 1993), and found more effective than ascorbic acid or PVP in reducing browning in *Dipterocarpus intricatus* (Linington, 1991).

WP basal medium supplemented with BA+GA₃ (8.88±0.72 µM) was found to be the best media for shoot multiplication. WP medium is reported to be the best and effective in several earlier studied in *Quercus* species, viz. *Q. leucotrichophora* and *Q. glauca* (Purohit *et al.*, 2002b), *Q. semecarpifolia* (Tamta *et al.*, 2008), *Q. rubra* (Vieitez *et al.*, 1993; Vengadesan and Pijut, 2009; Pandey and Tamta, 2015). The used cytokinin combination BA+GA₃ was also found suitable for *Q. rubra* (Vengadesan and Pijut, 2009), *Q. leucotrichophora* and *Q. glauca* (Purohit *et al.*, 2002b) and in *Quercus floribunda* (Purohit *et al.*, 2002a). During the study the alone cytokinin BA was

used; BA reported to be the best plant growth regulator in *Quercus shumardii* for shoot multiplication (Bennett and Davies, 1986). 20 μ M BA was found to be best for adventitious shoot induction and multiplication of individual shoots of *Q. semecarpifolia* (Tamta *et al.*, 2008). In contrast to multiplication from petiolar tube of *Q. semecarpifolia* the higher concentrations of BA were not found to be the best concentration although it induced highest number of shoots/bud but the length of shoots was reduced. Shoot multiplication was followed by rooting. Earlier study in *Q. semecarpifolia* suggests that IBA is the better responded auxin (root inducer) than NAA and a two step-rooting procedure resulted 100.0% rooting response, without the formation of basal callus (Tamta *et al.*, 2008). In present study two step method was used and about 88.89 percent rooting was recorded within 20 days of culture. Similar method of rooting has also been reported earlier in other *Quercus* species such as *Q. suber* (Manzanera and Pardos, 1990) and *Q. leucotrichophora* and *Q. glauca* (Purohit *et al.*, 2002b). During acclimatization the semi-rooted microshoots were not able to survive. The survival rate of microshoots was poor due to undeveloped rooting system to survive *ex vitro* conditions.

5. Conclusion

Results of present study are encouraging and could be useful in developing a rapid clonal propagation method for this important multipurpose tree species. However, the low rate of multiplication and poor survival rate of plantlets in the soil are the main challenges at the present. Therefore, to address the low regeneration and to improve survival rate of *Q. semecarpifolia* further research is required.

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Spent Mushroom Substrate of *Hypsizygus ulmarius*: A Novel Multifunctional Constituent for Mycorestoration and Mycoremediation

Padmavathi Tallapragada^{1,*} and Ranjini Ramesh²

¹Department of Microbiology, Centre for Post Graduate Studies, Jain University, 18/3, 9th Main, Jayanagar 3rd Block, Bangalore, India; ²Department of Environmental Science, Mount Carmel College, Autonomous, 58, Palace Road, Vasanthnagar, Bangalore, India;

*Correspondence: vam2010tpraviju@gmail.com / t.padmavathi@jainuniversity.ac.in; Tel: +91 9448533337

Abstract: 'Spent Mushroom Substrate' (SMS) is a composted growing medium that results from the mushroom growing process. The spent substrate remains after harvesting the mushrooms, which is entangled with innumerable mushroom threads (collectively referred as 'mycelia'), would have been biochemically modified by the mushroom enzymes into a simpler and more readily digestible form, which could then be used in 'mycorestoration' and 'mycoremediation'. Mushroom mycelia can produce a group of complex extracellular enzymes that can degrade and utilize the lignocellulosic wastes found in nature, which also reduces their potential for pollution. It has been revealed recently that mushroom mycelia can play a significant role in the restoration of damaged environments. Saprotrophic, endophytic, mycorrhizal and even parasitic fungi or mushrooms can be used in 'mycorestoration', which can be performed in four different ways: 'mycofiltration' (using mycelia to filter contaminated water), 'mycoforestry' (using mycelia to restore degraded forests), 'mycoremediation' (using mycelia to eliminate toxic wastes from soil and water) and 'mycopesticides' (using mycelia to control insect pests). These methods represent the potential to create a clean ecosystem, where no damage will be left after fungal implementation. 'Applied Mushroom Biology' can not only convert this huge amount of lignocellulosic wastes into human food but also can produce notable nutraceutical products, which have several health benefits and it is discussed in this chapter.

Keywords: Applied mushroom biology; *Hypsizygus ulmarius*; mycoremediation; mycorestoration; spent mushroom substrate

1. Introduction

Soils in the tropical regions of the world are fragile, contain very less organic matter and are prone to severe degradation, especially with increased deforestation and loss of topsoil. These attributes of tropical soils put constraints on food-crop production in these regions of high and dense human populations. In the last few decades, *Green Revolution* practices like using pesticides, synthetic fertilizers

and high-yielding varieties have been followed to overcome the constraints (Dalgaard *et al.*, 2003). With the help of these technologies, there has been a world-wide doubling of food crop production, but at the cost of environmental degradation of soil and water quality, reduction in biodiversity and suppression of ecosystem functions (Vance, 2001). Today, more than one billion people lack in food security and many village communities in these areas are continuously affected by a

steady reduction of food grains. In addition, the increase in industrialization has polluted our environment with chemicals and toxins of various kinds (Singh *et al.*, 2011). Most contaminated sites usually contain a mixture of non biodegradable persistent compounds, which increase the difficulties of remediation. This is due to the intensification of agriculture, range of crops grown and the diversity of manufacturing industries. The excess usage of chemical fertilizers has also contributed to the deterioration of the environment, with soil degradation, loss of soil fertility and agricultural productivity being the main consequences (Khan and Ishaq, 2011).

For improving the long-term sustainability of industry and agriculture, emphasis should be on the holistic management of natural resources. Microorganisms can control pollution and pests, maintain the fertility of soil and enhance plant growth, with no major adverse effects on the environment or other non-target organisms (Gomathi and Ambikapathy, 2011). These types of mechanisms rely on stimulating the growth of specific species of micro-organisms or mixtures of microflora native to the contaminated sites and are thus, able to remediate the area more easily and efficiently (Kumar *et al.*, 2010).

Recent research has favored the techniques of 'bioremediation' for cleaning up the above types of sites, as it is both environment-friendly and of relatively low-cost (Sasek, 2003). Bioremediation is the addition of biological agents, mainly microbes like yeast cells, fungi or bacteria to detoxify the contaminated soil and water. When fungi are specifically used, it is known as 'mycoremediation'. Ligninolytic basidiomycete fungi such as *Phanerochaete chrysosporium*, *Pleurotus ostreatus*, *Lentinula edodes*, etc. are well known mycoremediation agents. These microbes use the xenobiotics to be degraded as nutrients or as sources of energy (Tang *et al.*, 2007). Fungi play an important role in bioremediation, besides

being utilized extensively in industry, agriculture, medicine, food and textile industries (Prabhakaran *et al.*, 2011).

Hypsizygus ulmarius, the 'Elm Oyster Mushroom' (Figure 1), is a new variety of edible mushroom, developed by the Indian Institute of Horticultural Research (IIHR), Bangalore - www.iihr.res.in. It is a type of basidiomycete, also known as 'white rot fungi', of which there are about 1,400 known species. It can be commercially cultivated by solid-state fermentation method, using agricultural wastes such as paddy straw, coconut husk, tea and saw dust, among others. Mushroom cultivation is environment-friendly, in addition to providing a cost-effective source of food protein for vegetarians and a source of income for rural women (Ahmed *et al.*, 2009). Mushrooms are a good source of vitamins and minerals, while having low content of fats, carbohydrates and dietary fiber. With their nutritional value, mushrooms can reduce malnutrition in the rural poor to a large extent, and are also effective in reducing the occurrence of life-style diseases like hypercholesterolemia, hypertension, diabetes and cancer (Alam *et al.*, 2007).



Figure 1: *Hypsizygus ulmarius*: The Elm Oyster Mushroom, growing naturally on the bark of Elm trees (http://www.mushroomexpert.com/hypsizygus_ulmarius.html)

'Spent mushroom substrate' (SMS) is the by-product of mushroom

cultivation, and contains the fungal mycelium, fermented substrate, residues of inorganic nutrients and secreted enzymes such as ligno-cellulases, proteases and peroxidases (Medina *et al.*, 2009). SMS is also a rich source of carbon, nitrogen and other nutrients, and can be added to enhance crop growth and maintain soil fertility. It contains a consortium of bacteria and fungi which can mediate the formation and weathering of soil, nutrient and water mobilization, nitrogen fixation and denitrification processes. The fungal mycelium on the spent substrate is similar to 'Arbuscular mycorrhizal fungi' – AMF (Jonathan *et al.*, 2013). AM fungi especially function to mobilize water and phosphorus for plants (Manimegalai *et al.*, 2011). *Pleurotus florida* is a known 'mycorestoration agent', as it improves soil fertility by phosphate solubilization, increases aeration and water movements through soil and enhances plant growth (Kumar *et al.*, 2010).

White rot fungi are some of nature's most efficient lignin degraders from the microbial world, due to their ability to produce several kinds of lignin and phenol-degrading enzymes such as laccases and peroxidases. Laccases are copper-containing, glycosylated polyphenol oxidases. Their broad substrate specificity increases their significance in industrial and biotechnological applications such as biomechanical pulping of cellulosic matter, bleaching of pulp and degradation of dyes, chloro-phenols and a variety of xenobiotic and aromatic compounds, mainly by reduction of oxygen to water (Patel *et al.*, 2008). Extracellular laccase is usually secreted into the medium in small quantities. Its production is affected by typical fermentation factors such as media composition, carbon-nitrogen ratio of growth media, pH, temperature and diffusion of oxygen into the media (Revankar and Lele, 2006). Their production is stimulated by the addition of inducers such as phenolic and aromatic compounds like catechol, guaiacol, veratryl alcohol, ferru-

lic acid, 2,6-dimethoxyphenol, etc (Ikehata *et al.*, 2004).

Phenol, also known as carbolic acid, is a highly toxic element that is added in the manufacture of resins, herbicides and various other industrial processes (Amara and Salem, 2010). It is one of the most persistent chemicals, with high toxicity even at low concentrations, and is considered a 'priority pollutant' under the Environment Protection Act, 1986 (Chandrakant *et al.*, 2006). Phenols can be degraded by various white rot fungi like *P.florida*, *L.edodes* and *H.ulmarius* (Ranjini and Padmavathi, 2013; 2012).

Pleurotus florida, *Pleurotus ostreatus*, *Pleurotus flabellatus* and *Pleurotus sajor-caju* have also been studied for their potential in dye decolourization (Faraco *et al.*, 2009). Azo dyes are added in textile, pharmaceutical, cosmetic and food industries. After processing, almost forty percent of the dye is released into wastewater. This affects the aesthetics, transparency and oxygen levels of the receiving water, making it toxic (Ali *et al.*, 2008). Even at very low concentrations (<1 mg/L) in the effluent, they are visible and cause turbidity, especially red colour (Forgacs and Oros, 2004). Reactive dyes are significant because of their bright colour and low energy usage during application (Aksu, 2005). In exhausted dye baths and rinsing water, they are not recyclable or biodegradable.

Dyes can cause allergies, skin irritation and skin cancers, in addition to genetic mutations (Inbaraj *et al.*, 2002). Bacterial degradation of azo and reactive dyes usually occurs anaerobically, transforming them into carcinogenic intermediates; thus it is not considered suitable on a large scale (Manikandan *et al.*, 2012). Fungi, in aerobic conditions, can uptake and remove dyes without creating the above carcinogens. Physical adsorption onto the surface of spent mycelium, followed by enzymatic breakdown is the usual mechanism of fungal remediation of dyes (Zumriye and Karabayir, 2008). The effectiveness of fungi in bioremediation

of dye-polluted areas is because of their ability to penetrate and break the chemical structure of the dye molecules, as they have long hyphae and also their capability to degrade a wide range of dyes, especially anthraquinone and triphenylmethane dyes (Palmieri *et al.*, 2005). According to studies by Kodam *et al.*, (2005), azo dyes can be decolorized by *white-rot fungi*. Microbial decolourization uses both oxidative and reductive steps, oxidation brought about by peroxidases (lignin peroxidase, manganese peroxidase, versatile peroxidase, etc.) and laccases, usually found in these fungi for breaking down lignin, the main constituent of woody substrates (Gomaere and Govindwar, 2009).

The production of ligninolytic enzymes by *white rot fungi* is regulated to a great extent by the concentration and carbon-nitrogen sources added. Mikiashvili *et al.* (2005) proved this during his research on *Trametes versicolor*. It was observed that both the nature (i.e.) organic or inorganic source of nitrogen and concentration of nitrogen are important factors that regulate their secretion. Media with high nitrogen content produced higher quantity of laccase activity in *Lentinus edodes*, *Rigidoporus lignosus* and *Trametes pubescens*, while nitrogen-limited conditions enhance enzyme production in *Pycnoporus cinnabarinus*, *P. sanguineus* and *Phlebia radiata*. In some cases, high nitrogen content of the substrate suppresses enzyme activity. This occurs in substrates with high lignin content, correlated with a reduction in activity of peroxidase or phenol oxidase. However, substrates with low lignin content degrade well, which is correlated with increase of cellulase activity. This shows that *white-rot fungi* are more important in high-lignin substrates, where they are the primary organisms responsible for the secretion of phenol oxidase. This enzyme is suppressed by the addition of excess nitrogen. In low-lignin substrates, excess nitrogen stimulates a wider group of cellulose-degrading fungi and bacteria that

mainly produce cellulases (Singh *et al.*, 2002).

Carbon sources also have a regulating effect on enzyme secretion by *white rot fungi*. In *Phanerochaete chrysosporium*, ligninolytic genes are triggered by the depletion of carbon in the media (Wang *et al.*, 2008). In *Trametes pubescens*, significant laccase secretion occurs when glucose is reduced to a critical level. Glucose and cellobiose are good inducers of laccase, while fructose and cellulose inhibit it (Bettin *et al.*, 2008). The above observations indicate that carbohydrates can regulate laccase secretion in *white rots*. The source of carbon used is fungus-specific.

Other factors like pH, temperature, the presence of inducers and inhibitors have their own effects on enzyme secretion. The effect of extremes of pH may be due to the fact that it alters the three-dimensional structure of the enzymes. Higher temperatures can reduce enzyme production by drying of the substrate (Patel *et al.*, 2008). Surfactants like Tween 20 and Tween 80 are inhibitors, while veratryl alcohol, vanillic acid, ferulic acid, guaiacol and copper sulphate are inducers (Ikehata *et al.*, 2004).

The science of 'Applied Mushroom Biology' can provide solutions to the above environmental problems in the following ways:

- i. *Mushroom cultivation* - Production of inexpensive food protein (mushrooms) using agricultural by-products like paddy straw, coconut husk, tea, and saw dust, which also generates the *spent mushroom substrate* (SMS).
- ii. *Mycorestoration* – Addition of the above generated spent mushroom substrate to improve the fertility of marginal and contaminated soils by increasing soil aeration and the availability of phosphorus and potassium to plants.
- iii. *Mycoremediation* – Uptake and/ or degradation of environmental pollutants like phenol and dyes using

fungal enzymes present in the above generated spent substrate (Figure 2).

2. Mycorestoration of soil using spent mushroom substrate from mushroom cultivation

Spent mushroom substrate (SMS) also known as 'spent mushroom compost' (SMC), is generated as a by-product after the harvest of mushroom crop. Recently, it has been proposed to re-name it as 'post mushroom substrate' because it is not really 'spent' and has many uses remaining. The composition of spent mushroom substrate varies depending on the type of mushroom cultivated and the agricultural waste material used as substrate. It is an excellent source of humus, although much of its nitrogen content is used up by the growing mushrooms. Overall, it is a good source of the macro nutrients viz., nitrogen, phosphorus and potassium, in addition to having trace elements. This makes it suitable for supporting plant growth (Kulshreshtha and Sharma, 2014). This is also because it behaves similar to 'arbuscular mycorrhizal fungi' (AMF), associated with plant-growth-promoting rhizo-

bacteria (PGPR). The nitrogen content of SMS varies from 0.4-13.7% with a C: N ratio of 9 to 15: 1. It contains cations like K^+ , Na^+ , Ca^{2+} , Mg^{2+} ; and anions like Cl^- , NO_3^- , SO_4^{2-} ; all essential for optimal plant growth. It improves physical soil properties by decreasing its density, surface crust formation and diurnal temperature changes; in addition to increasing infiltration, aeration and water-retaining capacities. It maintains a high organic content of soil. It can be added singly or as a supplement to conventional bio-fertilizers, though it functions better in combination with other bio-fertilizers (Rinker and Kan Zeri, 2004).

Restoring a degraded or stressed soil using mycorrhizae and myco-bio-fertilizers is known as 'mycorestoration'. Humans have the ability to synergize mycorrhizae and use them for healing forest habitats that have suffered from stress, toxic waste or poor nutrition, making them critical to our mutual evolutionary survival (Stamets, 2006). Most plants also have associated with them diverse groups of plant-growth-promoting-fungi (PGPF) and AMF.

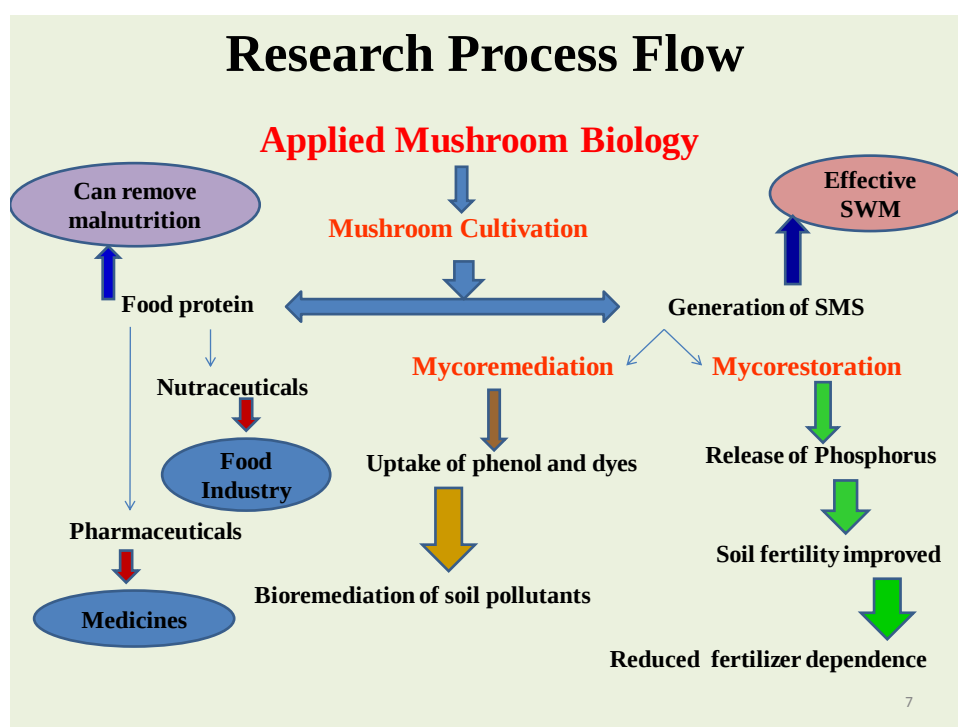


Figure 2: The science of applied mushroom biology.

2.1. The mushroom industry and mushroom cultivation

The ever-increasing demand for protein-rich vegetarian food and the inefficiency of conventional methods have resulted in the need to explore alternatives for low cost production of protein-rich food like mushrooms (Mukherjee and Nandi, 2004). The mushroom industry has a world production greater than 25 million tonnes. The largest producer is China, which cultivates more than 20 million tonnes and account for over 80% of the world's mushroom production (Li, 2012). Research has proved that production of 1 kg mushrooms will generate 5 kg of spent residual material called *spent mushroom substrate* or SMS. An average farm discards about 24 tonnes of SMS per month (Singh *et al.*, 2011). In Ireland, approximately 2,54,000 tonnes of SMS is generated each year (Barry *et al.*, 2012) and in The Netherlands, more than 8,00,000 tonnes (Oei and Albert, 2012). In some countries, waste management of SMS is a major problem faced by farmers and the government. The obvious solution is to increase the demand for SMS through exploration of new applications, being recycled and reused.

The spent substrate is a composted organic medium, made from renewable agricultural residues such as paddy straw, wheat straw, sawdust, sugarcane bagasse, hay, poultry manure, ground corncobs, cottonseed meal, cocoa shells, gypsum and other substances (Jordan *et al.*, 2008). Generally, each cultivation cycle lasts for 5 to 6 months, after which the spent substrate would be disposed. In Malaysia, an average farm producing 100 tonnes of fresh mushrooms per annum generates approximately 438 tonnes of SMS. The current disposal strategy of SMS in Malaysia is by burning, spreading on land, burying, composting with animal manure or land-filling.

2.2. Cultivation process for *hypsizygus ulmarius*

For obtaining the spent mushroom substrate (SMS) of *H.ulmarius*, it is cultivated on agricultural wastes such as paddy straw and coconut husk (Khan *et al.*, 2008), by solid-state fermentation method, as prescribed by Chang (1999).

2.2.1. Substrate preparation and sterilization

Crushed rice straw is used for cultivation. Straw is cut to 2-6 cm pieces, soaked overnight and autoclaved, at 121°C and 15 psi, while coconut husk is separated and soaked for 5-6 hours, then autoclaved as above.

2.2.2. Spawn rate

The quantity of spawn used for inoculation is 5% of its total weight (50 gm spawn for 1 kg substrate).

2.2.3. Spawning of substrate bag

The pasteurized substrate is filled into transparent perforated polyethylene bags; incubated at 23-25°C for 12 to 14 days. Mushrooms form around the edges of bag perforations and are harvested approximately 3 to 4 weeks later.

2.2.4. Spawn broadcasting

After spawning, the bags are moved to a room where temperature is around 18–20°C and relative humidity is close to 95-98%. The first 12-21 days are completed without artificial lighting. At the end of this period, 4 hours light is provided daily using fluorescent bulbs. At the time of formation of mushrooms, fresh air is let in to lower CO₂ levels.

Studies have shown that paddy straw is the preferred substrate for cultivation of *H.ulmarius*. However, coconut husk could be used as a supplement to enhance stipe length. Increasing stipe length can make picking the fruit during harvest much easier and thereby, increase its competitiveness in the commercial market. Rice straw, cotton waste, coir, baggase and banana leaves are all also considered suitable substrates for growing oyster mushrooms (Belewu and Belewu,

2005). The yield and quality depends on the C: N ratio, composition of vitamins, phytohormones, macro, and micro elements present in the substrate (Adenipekun and Gbolagade, 2006). The use of these agricultural wastes in mushroom cultivation provides a solution for their disposal, and effective solid waste management.

The cultivation of edible mushrooms offers one of the most feasible and economic methods for environment-friendly bio-conversion and disposal of agro-lignocellulose waste (Cohen *et al.*, 2002). The spent substrate after two or three harvests of mushrooms can be used for mycorestoration and mycoremediation studies.

2.3. Applications of spent mushroom substrate

The most significant applications of spent mushroom substrate (SMS) is its ability to increase and retain the organic content of soil or the potting medium (by increasing the release of major plant nutrients such as phosphorus and potassium); it also increases the porosity of soil by creating air spaces (due to the thread-like nature of fungal mycelia) – www.mushroom-sms.com.

Some of the other applications of SMS are as follows:

Consistency of quantity and quality – A consistent amount of SMS can be produced annually as mushrooms can be cultivated throughout the year with regulated temperature and humidity conditions. Consistent high quality can also be produced by standardizing the process and materials used for cultivation.

High water and nutrient retention capacity - Water and nutrient retention capacity of the soil increases by addition of SMS due to the filamentous nature of the mycelia that creates a network, similar to '*mycorrhizal fungi*', which traps the water molecules and also the released nutrients.

There is a reduction or complete absence in the growth of weeds- when SMS is added to soil.

SMS is already supplemented with nitrogen- along with its innate ability to release phosphorus and potassium, it makes the soil enriched in all the three major plant nutrients – nitrogen, phosphorus and potassium.

There is absence of heavy metals and toxins- in soil supplemented with SMS.

2.4. Use of spent mushroom substrate (SMS) of *Hypsizygus ulmarius* as a mycorestoration agent

The potential of the SMS of *H. ulmarius* has been studied for improving soil fertility and plant growth by the release of soil phosphorous, improving aeration and disease resistance. Phosphorous is one of the limiting factors for plant growth in most soils. SMS added singly increased soil phosphorus; with conventional bacterial biofertilizers such as *Azotobacter sp.* and fungal biofertilizers like *G.intraradices*, it enhances soil porosity, production of leaves, auxiliary buds and flowers; also root biomass, soil carbon and nitrogen. Hence, it is more beneficial when the SMS of *H.ulmarius* was used as a supplement to conventional fertilizers, rather than as a stand-alone bio-fertilizer. A lot of literature has indicated that there is a stimulatory effect of '*plant growth-promoting Rhizobacteria*' (PGPR) on the growth of plants, due to the presence of beneficial micro-organisms. However the mechanisms of this stimulation have not been discussed in detail in most of the literature. The modes of action that have been studied, are, however, as follows: Increased supply of nitrogen to the host by microbial nitrogen-fixation; increase in supply of phosphorus, sulphur and iron; increase in surface area of roots due to production of phytohormones and stimulation of mutualistic relationships between the host plant and other algae and fungi (Banerjee, 2006).

3. Mycoremediation of soil using white rot fungi

Bioremediation mainly depends on the ability of microorganisms like bacteria and fungi to produce enzymes that break down the pollutants to non-hazardous products. As bioremediation is effective only where environmental conditions permit microbial growth and activity, its usage often involves the manipulation of environmental parameters to allow faster microbial growth and degradation (Karigar and Rao, 2011). The limitations of bacterial growth are due to variations in pH, temperature, oxygen, soil organic matter, moisture and optimum level of nutrients, poor bioavailability of contaminants and the presence of toxins (Vidali, 2001). In this respect, fungi are far better adapted, as they exhibit high tolerance toward low pH and drought conditions, characteristic of contaminated and marginal lands. Most bioremediation systems operate under aerobic conditions, which is also a condition well suited for fungi (Leung, 2004).

Processes such as formation of insoluble metal oxalates, biosorption or chelation on the surface of polymers are used by fungi for removing pollutants from soil and water (Sasek, 2003). Some of the white rot fungi produce all the ligninolytic enzymes, while others produce only one or two of them. *Lentinus edodes* and *Pleurotus* spp. are fungi with important medicinal, biotechnological and environmental applications (Elisashvili *et al.*, 2008). They are capable of producing hydrolytic and oxidative enzymes like laccases and peroxidases, which are essential in breaking down the lignocellulosic biomass into low molecular weight compounds that support mycelial growth and fruiting (Reddy *et al.*, 2003).

3.1. Recovery of ligninolytic enzymes from spent mushroom substrate of white rot fungi

Enzymes reported in the literature are derived mainly from the mycelia of

macro-fungi grown in submerged fermentation. Enzymes can also be extracted from 'solid substrate fermentation technology'. Laccase is the most common enzyme isolated from the spent mycelium substrate (SMS) of *Agaricus bisporus* (Mayolo-Deloya *et al.*, 2009), *Pleurotus sajor-caju*, *P. ostreatus*, *Lentinus edodes*, *Flammulina velutipes*, *Hericium erinaceum* and *Hypsizygus ulmarius*. However, the productivity of lignin peroxidase (per microgram of SMS) was found to be the highest in the SMS of *P. sajor-caju*; it was twice, 22, 30 and 86-fold higher than that of β -glucosidase, laccase, xylanase and cellulase, respectively. Certain methods for extraction and purification of enzymes like laccase are dialysis, ultra-filtration, anion-exchange chromatography and gel filtration (Quaratino *et al.*, 2007). However, most of these experiments have been carried out using the fruiting bodies or mycelia of mushroom, not the spent substrate. The important parameters influencing enzyme yield are pH, temperature, extraction medium, incubation time, inoculum density and nitrogen source.

3.2. Mycoremediation of phenol using spent mushroom substrate

Phenol, if ingested, inhaled or absorbed through the skin, quickly penetrates the surface and causes severe irritation to the eyes and respiratory tract. It is potentially carcinogenic to humans (Muf-tah *et al.*, 2009). The *Hazardous Waste Management Rules 1989* permits only 5 kg of phenol per year for disposal. The actual quantities disposed are much greater. In addition, the present treatment methods are chemical intensive and further contaminate the environment. This has made it imperative that new non-chemical methods like *mycoremediation* are devised (Nuhoglu and Yalcin, 2005). There is a heightened concern over public health and environmental hazards due to the presence of organic toxins like phenols and dyes in waste water (Eriksson *et al.*, 2007). Phenols and azo dyes are well known for their bio-recalcitrant nature

and acute toxicity. They are being continuously introduced into ponds, lakes and rivers through from chemical and textile industries of different scale and category.

The major sources of phenol pollution are waste water coming from paint industries, pesticides, resin production and petrochemical industries. Phenols are considered as primary pollutants under directive 80/778/EC, since they are harmful to organisms even at very low concentrations (Calace *et al.*, 2002). The maximum concentration has been set at 0.5 mg/l for total phenols in drinking water, and individual concentration should be under 0.1 mg/l.

The main routes of exposure to phenol include breathing contaminated air; inhaling cigarette smoke, drinking water from contaminated surface or groundwater supplies, swallowing or inhaling products containing phenol or coming into contact with contaminated water and products containing phenol through bathing (Ahmaruzzaman, 2008).

The common method to detect intermediate products of phenol breakdown is extraction by organic solvent after esterification or acetylation, followed by gas chromatography and mass spectroscopy (GC-MS). However, this method has two disadvantages: (1) as the intermediates are mostly polar compounds, they dissolve easily in water and the non- or low-polar solvents don't get extracted completely (2) Esterification is suitable only for derivatizing acids, while acetylation only for hydroxylated compounds. In addition, it is not possible to detect these intermediates by *high-performance liquid chromatography* (HPLC). This method needs not only many calibration standards but takes a lot of time as there are several unknown compounds in the intermediates, and some compounds would have the same retention time (Guo *et al.*, 2006).

A possible degradation mechanism for phenol was given by Devi and Rajashekhar (2011). The hydroxyl radicals attack the phenol molecule leading to

the formation of dihydroxy benzene. Further degradation proceeds through the cleavage of dihydroxy benzene to give pent 2-enedioic acid and formaldehyde, in addition to benzoquinone and maleic acid. The decarboxylation of maleic acid and ring opening of hydroquinone result in the formation of oxalic acid. Decarboxylation of oxalic acid leads to the formation of carbon dioxide and water.

3.2.1. Application of spent mushroom substrate of white rot fungi in mycoremediation of phenolic compounds

The enzyme systems of 'white rot fungi' contain laccase, lignin peroxidase and manganese-dependent peroxidases which catalyses metabolism of many lignin-like structures, for example, PAHs and phenols (Eggen and Sasek, 2002).

Phenol oxidation using SMS from *A.bisporus* was reported and laccase was identified as the main enzyme responsible (Trejo-Hernandez *et al.*, 2001). Studies have also supported the idea of decontaminating phenolic compounds using SMS from cultivation of edible mushroom.

3.2.2. Phenol tolerance and degradation by spent mushroom substrate of *Hypsizygus ulmarius*

H.ulmarius tolerated and degraded phenol better under carbon and nitrogen limiting conditions of the growth medium. The optimum carbon sources for its growth were glucose, mannitol and cellulose, while ammonium nitrate, ammonium chloride and sodium nitrate were the optimum nitrogen sources. Peroxidase and manganese peroxidase were the enzymes secreted in maximum quantity during phenol degradation, followed by laccase (Ranjini and Padmavathi, 2012; 2013).

3.3 Mycoremediation of dyes using spent mushroom substrate

The majority of natural dyes are produced from plant sources like roots,

berries, bark, leaves, wood and also fungi and lichens. Azo compounds have aryl or alkyl functional groups, with vivid colors like reds, oranges and yellows. About 50% of dyes produced in the world are azo dyes (Perumal *et al.*, 2007). They are extremely recalcitrant and when released with the wastewater, remains in the water or soil and adversely impacts the photosynthetic ability of phytoplanktons in water. They interfere with the functioning of chlorophyll in the plankton, as they color the water and prevent the proper absorption of light (Duran and Esposito, 2000). Many methods have been tried for achieving decolorization of azo dyes in wastewater, but most of them like nanofiltration, specific coagulation, use of activated carbon and multiple effect evaporators are very expensive. Bio-treatment is a cheaper and environmentally better alternative (Olukanni *et al.*, 2006). Degradation by micro-organisms utilizes their enzymes, mainly laccases and peroxidases produced by few bacteria and white rot, brown rot and soft fungi (Duran and Esposito, 2000). Only these enzymes are found to be effective in breaking down the high structural integrity and variety of azo dyes. Decolourization and/or 'bio-adsorption' of dye-containing wastewater by dead or living biological matter (biomass), white-rot fungi and other microbial cultures are the subject of many studies reviewed in several recent papers (Aksu, 2005). In particular, these studies demonstrate that 'bio-sorbents' from suitable microbial biomass can be used for dye decolourization; this is because certain dyes have an affinity for binding with some microbial species. The use of biomass is becoming popular due to its availability in large quantities and cost-effectiveness. It is produced in fermentation processes to synthesize antibiotics and enzymes. Here, a large amount of by-products are generated, which can be used in bio-sorption of pollutants. Aksu and Tezer (2005) have shown the uptake of 588.2 mg of reactive black 5 per g of biomass of *Rhizopus arrhizus*. Chu and

Chen (2002 a, b) also reported the use of biomass for the removal of basic dyes.

Decolourization by living and dead microbial cells involves mechanisms such as surface adsorption, ion-exchange, complexation (coordination), complexation-chelation and micro-precipitation. Cell walls consist of polysaccharides, proteins and lipids and offer many functional groups for the above reactions. Dyes can interact with these active groups on the surface of the cell. The accumulation of dyes by biomass may involve a combination of active, metabolism-dependent and passive transport mechanisms. It starts with diffusion of the adsorbed solute to the surface of the microbial cell (O'Mahony *et al.*, 2002).

Laccase oxidizes the phenolic group of the azo dye with the participation of one electron, generating a phenoxy radical and then oxidizes it to a carbonium ion. A nucleophilic attack on the phenolic ring carbon bearing the azo linkage to produce 3-diazenyl-benzenesulfonic acid (III) and 1, 2-naphthoquinone then takes place (Camarero *et al.*, 2005). Phenolic radicals get oxidized further to yield oligomers. Under certain conditions, the C-C-formed dimers take part in coupling reactions to form extended quinines (Zille *et al.*, 2005).

In reactive dyes, the chromophore contains a substitute that is activated and allows the dye to directly react to the substrate surface. They are added for dyeing of cotton or flax.

The spent mushroom substrate (SMS) of *P.sajor-caju* offer an economical source of industrially important enzymes decolourize dyes (Singh *et al.*, 2002). Singh *et al.* (2011) has shown the decolorization of eight dyes (viz.) trypan blue, amido black, remazol brilliant blue R, bromophenol blue, crystal violet, methyl green, congo red and methylene blue using lignin peroxidase extracted from 5-month-aged SMS of *P. sajor-caju* coupled with veratryl alcohol as a redox mediator. Further, three azo group dyes, reactive black 5, reactive orange 16 and

disperse blue 79; two anthraquinone group dyes, disperse red 60 and disperse blue 56 and textile wastewater from all the five reactive and disperse dyes were also successfully decolourized by crude enzymes from SMS of *P.sajor-caju*. The mechanisms of enzymatic dye decolourization were most probably due to laccase and manganese peroxidase, as a recent study showed that under stimulation by malachite green, a triphenylmethane dye, the laccase and manganese peroxidase levels in the enzyme extracts of *P.ostreatus* were increased by 1.4 and 2.1-fold, respectively (Papinutti and Forchiassin, 2010). Thus, dyes can be removed, degraded and detoxified by enzymatic biological processes and also physical adsorption using SMS (Gao *et al.*, 2011). Use of SMS in bioremediation of dyes is both time saving and cost-effective.

3.3.1. Dye degradation using spent mushroom substrate (sms) of *hypsizygus ulmarius*

The SMS of *H.ulmarius* was effective in degrading three categories of dyes (viz.) azo (Congo red), heterocyclic (Methylene blue) and reactive (Solochrome black), with Methylene blue being most effectively decolourized, followed by Solochrome black and Congo red. Here, laccase was the enzyme secreted in greater quantity, followed by manganese peroxidase. Maximum secretion of both enzymes was observed during decolourization of Congo red (Ranjini and Padmavathi, 2015).

4. Conclusion

To conclude the effective management of agricultural wastes as a part of solid waste management is mushroom cultivation which utilizes agricultural wastes in an environment-friendly manner and solves their disposal problem, as they are generated in large amounts each year. The fertility of marginal soils increases in an environment-friendly manner - my-

corestoration. Using the spent mushroom substrate (SMS), a by-product of its cultivation, as a *bio-fertilizer*, either singly or in combination with conventional bio-fertilizers improves soil fertility, especially phosphorus, which is mostly insoluble and unavailable to plants. Phosphorus is one of the three macro nutrients of soil that can determine the yield of crops and agricultural income to farmers, and reduce their dependence on chemical fertilizers.

Soil contamination by phenolic compounds and dyes can be remedied using the spent mushroom substrate (SMS) of *H.ulmarius*. Phenolic waste is an integral part of biomedical and industrial waste. Present treatment methods are chemical-intensive and further pollute the environment.

The spent mushroom substrate (SMS) of *H. ulmarius* can also be used to decolorize dyes. Dyes color and pollute water bodies, kill aquatic organisms by increasing toxicity, and known to reduce of light and dissolved oxygen in water. The present treatment methods are mostly physico-chemical in nature.

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Biotechnology for Sustainability of Forests

Kumud Dubey¹ and Kesheo Prasad Dubey^{2,*}

¹Centre for Social Forestry and Eco-Rehabilitation, 3/1, Lajpat Rai Road, Allahabad, Uttar Pradesh, India; ²GM (East) Forest Corporation, Allahabad 24 B Agnipath, Forest Corporation, Allahabad, Uttar Pradesh, India; *Correspondence: dkumud@yahoo.com / dkesheo@yahoo.co.in; Tel.: +91 9415536077

Abstract: Biotechnology has various applications which could be utilized to enhance the sustainability in various sectors. Forest Biotechnology was started only during 1990s in the earnest sense. It encompasses structural and functional studies of genes and genomes (including development and application of genetic molecular markers); various methods of vegetative reproduction, and asexual insertion of genes into forest plant species. Traditionally, productivity of forests has been improved by introduction of new germplasm developed through tree genetics and breeding, as trees are strategically and efficiently able to utilize both horizontal as well as vertical space in an optimum way. The application of modern biotechnological tools and techniques that span the diverse fields of plant biology, genetic transformation and discovery of genes associated with complex multi-genic traits. Modern innovations have added an exclusively new dimension to forest tree improvement programs. However, forest biotechnology is still lagging behind because of longer time periods required for planning, investigation and field research, poor juvenile-mature correlations and multiplicity of selection criteria. But in the present scenario, with growing population, economic development, environmental degradation and Climate Change, the demand for more biomass production from trees for production of miscellaneous forest products and services, renewable energy alternatives to fossil fuels and carbon sequestration etc. is ever increasing. Currently, the major challenge is to maintain the original characteristics of pristine virgin forests for *in situ* biodiversity conservation for a sound natural genetic foundation. Genetic trees for pest and disease resistance will help in enhancing plantation survival, productivity and yield that would lead to eco-restoration of native tree species. This chapter highlights various application of biotechnology in forestry which could help in boosting sustainability.

Keywords: Bioreclamation; biotechnology; carbon sequestration; markers; micropropagation; phytoremediation

1. Introduction

Over the past few years, techniques of cell biology, genetic screening, and gene manipulation are being used to develop improved plant varieties. The term 'biotechnology' is coined for the collective use of these techniques. Biotechnology may be defined as "any technological application that uses biological systems, living organisms, or derivatives thereof, to make or modify products or

processes for specific use" (www.biodiv.org; FAO, 2001). It provides important tools for the sustainable development of agriculture, fisheries and forestry and can be of significant help in meeting the growing needs of population.

Rapid advancements have been and are being made in the scientific enquiry and investigation with regards to plant biotechnology throughout the universe today. Considerable progress has already been achieved in the field of med-

ical biotechnology, animal biotechnology and agricultural biotechnology. Research and applications of biotechnology in the field of forestry are also advancing rapidly. The term forest tree biotechnology started during 1980s. It involves a broad collection of tools for breeding, propagation and modern innovations that focus on a portion of a biological system (Yanchuk, 2001). As commonly used, forest tree biotechnology encompasses structural and functional studies of genes and genomes (including development and application of genetic markers); various methods of vegetative reproduction such as micropropagation, tissue culture, and somatic embryogenesis and genetic engineering, which is the physical manipulation and asexual insertion of genes into organisms (FAO, 2004). However, forest biotechnology is still aged behind because of inherent problems related with forestry, such as longer time periods for planning and investigation, poor juvenile- mature correlations (i.e. the characteristics features of young trees are not necessarily accurate indicators of those found in mature individuals), the multiplicity of selection criteria (e.g. timber quality, quantity, fuel wood, medicinal values, fodder etc.). Moreover, biotechnology in forestry requires collaborative and integrated application of knowledge and techniques drawn from several diverse disciplines like agriculture, silviculture, genetics, microbiology, molecular biology, plant physiology etc. Biotechnology applications in forestry are a growing area of interest. Initial applications of forest tree biotechnology targeted to improved productivity and quality of plantation forests. Such use, with appropriate social controls, can help to reduce impacts on natural forest ecosystems from timber harvest-related perturbations (Sedjo, 2001). Through biotechnology faster growing trees can be produced thereby decreasing the growth as well as harvesting period. Biotechnology can also reduce threats to tree health by introducing the traits that confer resistance to different

diseases. Improvements through tree biotechnology may also improve weed control enabling young trees to compete with weeds in natural ecosystem. Trees genetically engineered for pest resistance may promote plantation survival and yield, and also lead to restoration of native tree species. Other potential benefits include enhancing the ability of trees to tolerate abiotic stress; restoring contaminated sites through phytoremediation; facilitating weed control using more environmentally benign treatments; producing new industrial products; modifying biomass chemistry to improve pulp and biofuels production; and improving carbon sequestration to mitigate greenhouse gas emissions. In addition, biotechnology, especially Genetic Engineering methods, offers unique and important tools to conduct research to identify the biological mechanisms for control of many ecologically and economically significant traits. The application of biotechnology offers a great potential to hasten the pace of tree improvement for desired need and can improve the productivity of forest and environment.

Most of the biotechnologies used in forestry today involve vegetative reproduction through tissue culture and molecular marker applications. However, Genetically Engineered Plants are also likely to play a major role in forestry.

2. Application of biotechnology in forestry

In the present scenario, with growing population and economic development, the demand of wood products is increasing which will increase the demand for more biomass production from trees in the future for carbon sequestration and to meet the demand for forestry products and renewable energy alternatives to fossil fuels (Scholes and Noble, 2001). Forestry products are the third most valuable commodity after oil and gas. Trees supply the bulk of fiber for pulp, paper, packaging and building

needs. Three billion people depend on wood for fuel. So we must harvest wood. But forests also are an essential component of our ecology. It is essential to enhance the productivity of plantation forests in order to meet the ever increasing future world demand for wood and wood-based products in a sustainable manner that preserves natural forests and biodiversity (Scholes and Noble, 2001). The major challenge of foresters today is to maintain the natural characteristics of forests while meeting society's need for products produced from trees. Implementation of improved silvicultural techniques and forest management practices are being used to manage the forest in sustainable manner. Productivity of the forest has also been improved by the introduction of new germplasm developed through genetics and breeding efforts for tree species. The application of new biotechnological tools and techniques that span the fields of plant biology, genetic transformation and discovery of genes associated with complex multigenic traits along with genetic engineering have added a new dimension to forest tree improvement programs. Significant progress has been made during the past few years in the area of plant regeneration via organogenesis and somatic embryogenesis (SE) for economically important tree species. These advances have not only helped the development of efficient gene transfer techniques but also have opened up avenues for using new high growth performance clonally replicated planting stocks in forest plantations. Advancements in gene cloning and genomics technology in forest trees have enabled the discovery and introduction of value-added traits for wood quality and resistance to biotic and abiotic stresses into improved genotypes. One of the greatest challenges today is the ability to extend this technology to the most elite germplasm, such that it becomes an economically feasible means for large-scale production and delivery of improved planting stock. Commercialization of

such newly developed planting stocks as new varieties generated through clonal propagation and advanced breeding programs or as transgenic trees with high-value traits, is expected in the near future, and these trees will enhance the quality and productivity of our plantation forests. The application of plant biotechnology techniques promises potentially significant genetic improvement of forestry species and may become very attractive in view of several constraints traditionally imposed by the long life cycles and physical size of trees (Schuch, 1991). The potential applications of biotechnology in forestry may be classified in three broad categories: Plant Culture, Plant Protection, and Plant Utilization.

2.1. Plant tissue culture

Genetic improvement in plantation forestry relies significantly on conventional breeding techniques which have been extensively used to improve various characteristics in forest trees such as growth and form, volume yield, resistance to pathogens and quality of the end product (Walter *et al.*, 1998). Traditional breeding techniques involve identification of superior trees with desired traits and selection of the offspring having desired traits. It was the major technique used for the planting stock improvement in 1970s. In the 1990s, biotechnology was introduced in forestry in earnest (Sedjo, 2001). Forest biotechnology offers new perspectives in the genetic improvements of forest trees through tissue culture and genetic engineering. Plant tissue culture broadly refers to the techniques of growing plant tissues or parts on a nutrient medium containing minerals, sugars, vitamins and plant hormones, all under sterilized conditions. It is a basic technique to be used for multiplying elite clonal germplasm or genetically engineered plants of forestry species. Plant tissue culture of forestry species are aimed to fulfill following objectives:

- i. Micro-Propagation: To develop protocol for micro propagation of im-

- portant forestry species and improve vegetative propagation procedures including tissue culture procedures.
- ii. Genetically engineering: To identify, characterize and isolate genes of interest and genetically engineer tree seedlings to acquire desirable traits and qualities.
 - iii. To develop molecular markers to help aid in progeny selection.

2.1.1. Micro-propagation of forestry species

In India, forestry biotechnologies involve vegetative propagation mostly through tissue culture. The method offers substantial advantages over plants obtained through seed origin. It controls genetic diversity, ensures greater uniformity and high proportion of non-additive genetic variance, eliminates inbreds, provides clones of desired traits and helps in predicting the yield of plantations. Vegetative propagation allows cloning of superior lines and prevents the loss of desirable traits and the uncertainties associated with sexual reproduction. In addition, the ability to propagate transformed cells (cells to which DNA has been introduced via the techniques of biotechnology viz. Genetic Transformation) and to regenerate plants from cultured cells, micropropagation is prerequisite to genetic engineering. Micro-propagation is a term used here to describe methods of *in vitro* vegetative multiplication including rooted micro-cuttings, organogenesis and somatic embryogenesis. Micro-propagation is aimed at cloning superior individuals or at 'bulk' (in mixture) propagating new genotypes with high genetic potential but available in limited quantities (such as materials obtained by controlled pollination). Vegetative propagation bypasses the genetic mixing associated with sexual reproduction. Researches are mainly focused on the improvement of rooting procedures for cuttings and micro-propagation through organogenesis and somatic embryogenesis.

Micropropagation by microcuttings consists of mass producing vegetative copies of desired genotypes by either axillary or adventitious budding. Micropropagation by microcuttings is carried out on more than twenty species including *Populus alba*, *P. deltoides*, *P. tremula* and *Populus* hybrids in Germany and India (Cornu, 1994), Spain (Bueno *et al.*, 2003) and Lithuania (Kuusiene, 2002); *Eucalyptus camaldulensis*, *E. globulus*, *E. grandis*, *E. nitens*, *E. tereticornis* and *E. urophylla* in South Africa, Spain and Portugal (Watt *et al.*, 2003), India (Watt *et al.*, 2003; Nadgauda in press), Vietnam and Thailand (O. Monteuiis personal observation) and Australia; *Acacia mangium*, *A. melanoxylon*, *A. mangium* × *A. auriculiformis* in Malaysia and South Africa (Galiana *et al.*, 2003; Monteuiis *et al.*, 2003; Quoirin, 2003); *Tectona grandis* in India (Bonga and Von Aderkas, 1992; Nicodemus *et al.*, 2001; Nadgauda, in press), Vietnam, Brazil and Indonesia (O. Monteuiis personal observation), Thailand (Kjaer *et al.*, 2000), Costa Rica (Schmincke, 2000), Malaysia (Goh & Monteuiis, 2001); *Pinus* sp. in United States (Rahman *et al.*, 2003); *Anogeissus latifolia* and *A. pendula* in India (Saxena and Dhawan, 2001); *Gmelina arborea*, *Artocarpus chaplasha*, *A. heterophyllus*, *Azadirachta indica* and *Elaeocarpus robustus* in Bangladesh (Sarker *et al.*, 1997; Roy *et al.*, 1998).

Somatic embryogenesis, or production of embryos from somatic cells, is in fact a cloning technique, as opposed to zygotic embryogenesis, in which germinal cells give rise to seedlings that are all genetically different. Somatic embryogenesis is a type of plant tissue culture that starts with a piece of donor plant and forms new embryos. In the right culture conditions, embryos could be developed from the somatic cells. The process of somatic embryogenesis derives usually from callus formation induced by applying cytokinetic or auxinic exogenous growth regulators to very juvenile plant tissues. In the most favourable situations,

some undifferentiated cells of these calli can evolve into somatic embryos characterized similarly to zygotic embryos, by a shoot–root bipolar structure. This basically distinguishes somatic embryogenesis from microcuttings consisting first of a shoot from which an adventitious root must subsequently develop. Somatic embryogenesis offers the advantage of rapid embryo multiplication in a small space. Somatic embryogenesis is of specific interest in the long term because it has the potential for producing, inexpensively, large numbers of clones that can be propagated as artificial seeds. In the case of conifers, somatic embryogenesis, especially when derived from a single cell, seems the most suitable regeneration and propagation technique. In broad-leaved species, vegetative propagation of genetically engineered materials is likely to use a combination of micropropagation and rooted stem cuttings, at least in the beginning. The advantages of somatic embryogenesis in comparison with micropropagation through microcuttings are especially with regard to multiplication rate and genetic modification applications. Somatic embryogenesis is preferred to micropropagate conifers (Sutton, 2002; Lelu-Walter and Harvengt, 2004). However, there are still serious obstacles to large-scale operational application of somatic embryogenesis to forest trees, for example:

- Only few species and within these species, only few genotypes can produce somatic embryos.
- Success has been obtained with few exceptions, mainly with juvenile tissues coming for instance from immature zygotic embryos.
- There are risks that somaclonal variation may decrease the value of the genotypes produced by somatic embryogenesis, resulting in a considerable waste of time, material and money. True-to-typeness, particularly, may remain a problem for certain genotypes and efforts are still needed for optimizing this technique to make

it more reliable, especially when using mature selected genotypes.

Organogenesis or the creation of plantlets from tissues such as cotyledons is not common and rarely used in forestry. Organogenesis and somatic embryogenesis have been achieved in a large number of woody plant species. However, in a majority of tree species, micropropagation has been carried out by employing juvenile explants, for example embryos, cotyledons and shoots tips from seedlings. Clonal propagation from mature trees, in particular conifers, is still very difficult by tissue culture and remains a challenging biotechnological problem. Micro propagation of commercial forest tree species is a major research and development goal.

2.1.2. Genetic engineering

Future prospects for genetic engineering of forest trees are high. Through the use of genetic engineering techniques, individual genes of interest may be isolated from the donor organism and transferred to a target microbe, animal, or plant cell. Forest trees have generally been more difficult to work with mainly due to their long generation times and life cycles. Through genetic engineering, foreign genes can be transferred to a forest tree resulting in faster tree improvement and unique gene combinations which cannot be achieved by traditional tree breeding. The successful genetic engineering of a forest tree requires four factors:

- i. A desirable gene must be identified and isolated from a donor organism.
- ii. A plant regeneration protocol from single cells or a small group of cells.
- iii. A mechanism for inserting or transferring foreign DNA into a target cell is required.
- iv. Additional DNA sequences for regulating the target gene, e.g. a promoter is necessary to cause the target gene to function in the

proper tissue when and where desired.

Considerable research is focused on identifying, characterizing and isolating genes in trees that are responsible for traits of particular interest, including stress responses viz. drought, frost and water-logging etc., disease tolerance and or resistance growth characteristics, wood quality and flowering. The examples of traits for which genes are being sought in commercial forest trees include lignin composition, disease and insect resistance, dormancy, cold hardiness and growth rate etc. Genes of interest can be divided into two categories. First, genes those are responsible in governing agronomic traits or growth of trees. These genes are expected to lower the cost of wood. This category includes genes for disease and pest resistance or tolerance of environmental stresses. Genes that help a crop to grow more efficiently, such as herbicide tolerance, also fall into this group. A second category includes genes for value-added traits that improve production efficiency, product quality, or product value and are expected to increase the value of wood or quantity and quality of active bio-chemicals. For instance, genes for reduced lignin content or lignin type which are more easily removed during pulping fall into this category. Genes responsible for improved fiber characteristics would also be included here. Several value-added traits are being actively studied in order to isolate valuable genes.

Several gene transfer systems are available for movement of foreign DNA into target plants. The most common method uses *Agrobacterium tumefaciens*. This soil-borne bacterium is able to naturally genetically engineer plants to create an environment in which the bacterium can thrive. Molecular biologists have altered this organism to insert target genes into plants without causing plant disease. Other methods for gene transfer in forest trees include particle bombardment, elec-

troporation and polyethylene glycol. Most successful work on genetic transformation of forest tree species genomes so far has been obtained by using juvenile material, for example from an explants produced from juvenile tissues which have much higher regeneration capacities than older material. Successful genome modification reports of adult selected plant material are very rare, except in poplars precisely because of its greater capacity to regenerate.

Transformation of a number of tree species by genetically engineering trees has been reported. *Populus* spp. serves as the model for much of the research because techniques for genetic engineering and micropropagating are relatively advanced. However, research in this area is still modest because several technical problems have to be solved.

Limitations to the broad use of genetic engineering to improve forest trees include primarily the following facts:

- i. Only a few genes for specific traits of commercial interest have been identified,
- ii. Culturing cells of many tree species is difficult and
- iii. Regenerating whole plants from cultured cells of commercial tree species has met with only limited success.

Those biochemical activities that are specific to woody plants or even to single species or varieties of trees, such as wood fiber formation and resistance (or susceptibility) to specific insects or diseases often must be studied in the tree species of interest. It is important to note, however, that much of the research on non-forest plants is relevant to commercial forest trees. For example, the research underway on cellulose biosynthesis in cotton plants is focused on uncovering enzymes and genes for which similar counterparts can be anticipated in trees. The same is true for photosynthesis research on spinach. Major research efforts are underway in many laboratories on the small plant *Arabidopsis thaliana*

because it completes its life cycle in 6 weeks. Funds for much basic biochemical research are more effectively spent on plants other than forest trees.

2.1.3. Molecular markers

A serious problem today is in identifying desired progeny before they get too old to propagate vegetatively, by root cuttings, for example. Marker-assisted selection (MAS) has given further impetus to tree breeding and selection. Molecular markers allow rapid identification of the gene or genes of interest in minute samples. Molecular markers are genetically linked to a given allele on a given locus and can therefore, be used to predict the presence of the allele with great accuracy (FAO, 2004). Biochemical and molecular markers play a significant role in many forest biotechnology activities for the selection of desired progeny. Research aimed at developing molecular markers for screening is accelerating. Marker applications for fingerprinting and paternity analysis been used for characterization of genetic diversity. Isozymes, randomly amplified polymorphic DNAs (RAPDs) and restriction fragment length polymorphisms (RFLPs) have been widely used for genetic diversity and mapping studies, though the current trend favours microsatellites (nuclear and cytoplasmic) and AFLPs (amplified fragment length polymorphisms). Currently, ESTs (expressed sequence tags) and SNPs (single nucleotide polymorphisms) represent the most active area of marker development (FAO, 2004).

3. Plant protection

Forest trees are affected by a large number of different insect pests and diseases, the latter caused by fungi, bacteria and viruses. Biotechnology offers a powerful approach to mitigating the damage caused by insects and diseases as well as by environmental stressors. Insects and diseases probably cause great damage to Indian forests; environmental stressors

like drought and water logging add to it, quite substantially. Shoot borer in *Shorea robusta* and *Fusarium* wilt of *Dalbergia sissoo* greatly damage these important species of timber. Through the application of Biotechnology threats to tree health can be reduced. Research is showing promise in the introduction of traits that confer resistance to pests and pathogens that weaken or cause heavy mortality of trees. Improvements through tree biotechnology may also improve weed control enabling young trees to get a head start over nutrient-robbing competitors. The research falls into two categories: (a) direct protection through control of insects and diseases and (b) indirect protection through development of resistant tree varieties.

3.1. Direct protection

The biotechnology product “Bt toxin” is already being used commercially in agriculture and forestry. Produced by the bacterium *Bacillus thuringiensis*, this toxin is effective against lepidopterous insects. One biological approach is to use pheromones to attract the insects to a central point for control. Techniques of molecular biology apparently have not yet been applied to these pests. Direct bio-control of rusts and other fungal, bacterial and viral diseases of trees is labour intensive and costly at best.

3.2. Indirect protection

Trees have a wide variety of natural defenses against insects and diseases, which is why most microbes, insects and viruses do not cause tree damage. Considerable research is underway to identify genes that confer resistance to fusiform rust and white pine blister rust in resistant varieties and species of pines. When these genes have been identified, they can theoretically be inserted into susceptible trees to provide resistance. This is fairly long-range research. In the near term, the identification of markers for these resistance genes will allow marker-assisted tree breeding and asexual progeny selection to

hasten the development of resistant lines of trees. Stress tolerance genes are also being sought. Another biotechnological approach to indirectly controlling insects and diseases in trees is to insert foreign genes that confer resistance. Thus, several tree species have been transformed with insect-controlling genes such that for Bt toxin.

The production of insect resistant plants via genetic engineering has generally taken one of two approaches. The first approach makes use the Bt toxin derived from *Bacillus thuringiensis*. This toxin damages the digestive mechanisms of the larvae that feed upon it. The toxin specifically affects insects belonging to the lepidopteran, dipteran and coleopteran orders of insects, which include a number of major herbivores of forest tree species. The Bt toxin gene was first used to transform hybrid poplars by McCown *et al.*, (1991) via direct (biolistics) gene transfer. The introduction of the Bt toxin gene resulted in a significant reduction in forest tent caterpillar (*Malacosoma disstria*) survival and growth rates of the gypsy moth larvae (*Lymantria dispar*). Herbicide resistant crops have been one of the major products of the first generation of agricultural biotechnology. They are intended to reduce weed control costs, increase control flexibility, facilitate the use of low-tillage (and thus reduced erosion) cropping systems and enable broad-spectrum and environmentally benign herbicides to be more readily employed. The first successful transformation of a woody species was reported in *Populus alba* × *P. grandidentata* using *Agrobacterium tumefaciens* (Fillatti *et al.*, 1987). Transgenic hybrid poplars, with a reduced sensitivity to glyphosate, an extensively used broad-spectrum herbicide, were produced.

4. Plant utilization

Genetic transformation of tree for its commercial end-use can be achieved through biotechnology. For example lignin composition can be quantitatively and

qualitatively modified through genetic engineering for expected financial gains from pulp processing improvements. Lignins, which enhance cell wall mechanical properties and hardness, are difficult to process and are a significant limitation in processing wood into paper pulp by chemical treatment. Genetic transformation to modify lignin characteristics is a key research feature on species used in the paper industry. The aim is to regulate the activity of key enzymes involved in the lignin biosynthesis pathway (Jouanin *et al.*, 2000; Le *et al.*, 2003).

5. Benefits of biotechnology in forestry

Forestry is in the take-of stage today as biotechnology is introduced into its several operations bringing promising developmental changes having tremendous potential. The use of biotechnology for tree improvement can bring economic, social and environmental benefits. Biotechnology has many useful applications in forestry viz. lignin reduction, fiber modification, pest and disease resistance, bio control methods against weeds, pests and diseases, cellulose content enhancement, development of cold and hot tolerant species of a desired species, modification of biomass chemistry to improve biofuels and pulp production, phyto-remediation of problem contaminated sites viz. arid, ravine, saline and usar sites, improving Carbon sequestration potential to mitigate greenhouse gas emissions and sterility, which is an important factor to prevent modified genes from “leaking” into the natural environment. The short term economic gains from the introduction of biotechnology to forestry will be lower costs and increased availability of wood and wood products at shorter rotation than usual. Innovations in forest biotechnology have the potential to address important environmental issues, including the rehabilitation of habitats altered by diseases like the Sal Borer Attack in Sal Forests, Drying of Sheesham, or invasive exotics. Moreover, the increased productivity of

biotechnology driven tree plantations may free huge chunks of natural or primary forest areas from ever increasing pressures to supply industrial wood and thus improve their ability to maintain and preserve biodiversity. And as trees are genetically modified to be able to grow in previously unsuitable areas such as arid or Usar lands and saline soils the newly created forests could not only produce more wood but also enhance watershed protection and sequester carbon for climate change mitigation.

5.1. Benefits of biotechnology

5.1.1. Economic benefits

Introduction of any technology for the consumer simply means that relative prices of the desired goods fall compared to that which would have been in the absence of the particular technological innovation. In other words, technology features increased productivity, that is, enhanced output per unit of input. Alternatively, technology can be either cost (input) reducing or yield (output) enhancing. For society, more output for the equivalent expenditure of inputs means societal increase in efficiency. Incidentally, Plantation Forestry has received some success in recent decades because of its associated cost-reducing technology that has given wood from planted forests a competitive price advantage over that harvested from natural forests. The potential applications of immediate interest of cost-reducing biotechnology to forestry are increased wood production, improved tree form and wood quality, enhanced survival, quicker growth rates and enhanced resistance to insects, diseases and herbicides. In addition, production and processing costs of wood or chips could be reduced as well as financial and environmental costs for pulping.

Paper production, for instance, requires fiber with adequate strength to allow sheets to be produced on high-speed machines, an attribute determined by the wood fiber characteristics. Therefore, in pulp making for paper production,

desirable traits would be the easy breakdown of wood fibers and the removal of lignin during chemical processing. Through Biotechnology, the raw material for paper production can be customized to meet the requirements of producers thereby wood values increase to that extent.

5.1.2. Environmental benefits

Forestry Biotechnology may also be used to create a number of desirable environmental outputs (Table 1).

Table 1: Utilization of biotechnology for environmental benefits

No.	Biotechnological innovation	Environmental output
1	Cheaper plantation wood substitutes for wood from natural forests	Pressure to log primary forests can be reduced
2	Trees are genetically modified to grow in arid, usar or saline conditions	Ecological forests can be established on degraded lands
3	Trees are genetically modified to adapt to traditionally unsuitable sites	Carbon-sequestering forests can be established on sites previously not suitable for forestry
4	Cold-tolerant species of a desired genus are developed	The altitudinal and geographical range of desirable tree species can be extended

Trees genetically engineered for pest and disease resistance may promote plantation survival and yield and also lead to eco-restoration of native tree species like Sal. Biotechnology has the potential to enhance the ability of trees to tolerate abiotic stresses; restoration of contaminated sites through phytoremediation; facilitation of weed control using more environmentally safe treatments; modification of biomass chemistry to improve

pulp and biofuels production; and improving carbon sequestration to mitigate greenhouse gas emissions. Biotechnology also offers the potential to assist in ecosystem restoration and repair. Similarly, biotechnology may help deal with invasive exotics, which have in many places threatened locally and easily available indigenous species. Modified tree species also prove useful in providing ecological and environmental services in areas where trees now have difficulty in surviving, such as arid or drought-prone areas and areas with saline soil or frost zones and industrial waste effluents viz. Dairy waste disposal sites, Aluminium waste effluents-Red Mud, Fly Ash Ponds in Thermal Power Plants etc.. Another contemporary and very important application of biotechnology from the present Climate Change scenario involves creation of biological sinks, a potential tool to mitigate the build-up of greenhouse gases associated with global warming. Land Areas and Wetlands not currently forested could grow carbon-sequestering plantations of Genetically Modified transgenic trees (GM Trees).

The most threatening cost of Biotechnology is the after-effect of transgenic plants on the natural ecosystem, when there would be genetic exchange(s) between domestic and wild populations. In cases where plantation tree species are exotic, genetic “outcrossing” to the natural environment would not be a factor. Where genetic exchange could be a problem, planting sterile trees or varieties with reduced or delayed flowering would lessen the likelihood of their “escape” to the natural environment. In the case of the herbicide-tolerant gene, the consequences of release into the wild are probably small. Herbicides are unlikely to be applied to most of the natural environment, and where necessary, other types could be used to which the escaped genes do not confer tolerance. In the long term, the herbicide in question will almost surely be replaced periodically in the normal course of product change and develop-

ment. Thus, the presence of that modified gene in the natural environment appears unlikely to constitute any serious environmental problem, either short- or long-term.

For qualitative genes that affect tree form or wood fibre characteristics, release into the natural environment is unlikely to provide a competitive advantage in survival and therefore, unlikely to have significant or adverse consequences on the ecology. However, the consequences could be different if a survival gene is involved. For example, the introduction of *Bacillus thuringiensis* (Bt) gene makes a plant toxic to certain pests. The release into the wild of such a gene could constitute a major problem if it altered the comparative competitive position of wild vegetation vis-a-vis those pests. However, the seriousness of this problem depends on the probability of the transfer of a survival gene into the wild, the scale of the transfer and the comparative change in the competitive ecological balance within the natural habitat. Since pests adapt via natural selection to modified genes, the long-term impact of the release of the modified gene into the natural environment will be mitigated. Subsequently, wild populations would gradually become resistant to the *Bacillus thuringiensis* (Bt) gene, thereby undermining its long-term effectiveness against those pests.

Transgenic biotechnology has become controversial in agriculture. Some of those controversies appear to be spilling over to forestry too. The controversy revolves around a number of issues. One such issue involves the effects of biotechnology, particularly the introduction of transgenic plants on human health. The food safety issue is not generally raised for plants such as forest trees, which are not usually a food source. However, cellulose is increasingly being used as filler in food products, and the food safety issue could become a concern, subsequently to be encountered by the forest biotechnologists.

Therefore, the most visible costs of forest biotechnology are those associated with Modified Genetically Engineered (GE) trees which include among others, effects of the newly acquired traits on forest ecosystem structure and function; unintended consequences of inserted genes on tree and forest biology; reliability of the newly encoded traits to produce the desired outcomes; and persistence and potential impacts of the introduced genes in native populations through the dispersal of pollen, seeds or vegetative propagules (Frankenhuyzen and Beardmore, 2004). Other apparent risks from biotechnology are associated with loss of genetic diversity from vegetatively propagating a small number of highly selected varieties. Another serious disadvantage of vegetative propagation of highly selected varieties may ultimately result in vulnerability to insect and microbial pests and also to stressful climatic events.

6. Concluding remarks

Before taking any new biotechnological strides in the forestry sector, owing to the complexity of forest ecosystems, several ecological benefits, costs and risks and the effectiveness of risk management practices should be completely evaluated through careful review of scientific literature and well-designed field experiments.

The benefits of biotechnology in forestry go beyond economic advantages including increased production, lower costs to consumers and trees modified for easy wood processing or specific production objectives etc. to environmental surpluses viz. preservation of biodiversity, eco rehabilitation of degraded lands, eco restoration of contaminated sites and mitigation of global warming etc.. But biotechnological innovations raise immediate serious concerns about biosafety and the effects of transgenic plants on the resistance of pathogens and on the natural ecosystem too, particularly the question

of genetic exchange between genetically modified trees and wild populations.

Genetic engineering, by identifying and isolating specific genes to serve a particular purpose, can allow a novel trait to be transferred to any genotype in a single generation with little or no alteration to its other genetic properties. Thus, if there is to be a fertile crescent in forestry, it is to be found in the Biotechnology Laboratories and Field Experiments that operate at the interface of basic plant molecular genetics and forestry.

Genetic engineering can provide exceptional particular genotypes that can be characterised by corresponding specific molecular markers and subsequently integrated in the clonal forestry and breeding programs. Further progress in this area will depend on reliable regeneration and automation for mass production of selected particular genotypes. It is expected that future research in biotechnology will provide insight into the control of maturation and rejuvenation, directed gene transfer, genome structure, gene sequence and basic mechanisms involved in growth and differentiation of trees. Biotechnology is going to play an important role in the 21st century in boosting sustainability of Forests.

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Biotechnological Approaches for Conservation and Sustainable Supply of Medicinal Plants

Sagar Satish Datir^{1,*} and Subhash Janardhan Bhore²

¹Department of Biotechnology, Savitribai Phule Pune University, Pune – 411007, MS, India; ²Department of Biotechnology, Faculty of Applied Sciences, AIMST University, Bedong-Semeling Road, 08100, Bedong, Kedah Darul Aman, Malaysia; subhashbhore@gmail.com / subhash@aimst.edu.my (SJB);

*Correspondence: datirsagar2007@gmail.com; Tel.: +91 8412013810

Abstract: Food and medicines are integral part of human life. Continuously increasing global population and food demand has created an alarm about sustainable use of natural resources. Due to adverse environmental conditions such as drought, salinity, temperature and pathogens, it is very challenging to achieve high yield with current agricultural practices. Due to deterioration of food quality and unpredictable environmental conditions, there is a major public health concern about various diseases. Plant-derived compounds are playing significant role in combating various human diseases since prehistoric times and therefore, there is an increasing demand for production of plant-derived secondary metabolites. However, due to mismanagement of natural resources and faulty agricultural practices, several medicinal plant species have become rare, vulnerable and endangered. Hence, alternative strategies are needed to protect medicinally important plant species. Biotechnology has become a center of attraction due to its innumerable advantages in agriculture, pharmaceuticals, forestry and food sectors. In recent years, plant-derived compounds (also called as natural compounds) are widely studied and biotechnological tools such as, *in vitro* propagation, transgenic for secondary metabolite production and cryopreservation not only provided alternative but also offer sustainable approaches towards conservation of medicinally important plant species. This brief review highlights various biotechnological approaches for conservation and sustainable supply of medicinal plants. Achievements, challenges and perspectives on *in vitro* propagation for the conservation of medicinal plants are also highlighted.

Keywords: Biotechnology; conservation; medicinal plants; plant tissue culture; secondary metabolites; sustainable development

1. Introduction

Climate change, biotic and abiotic stress, depletion of natural resources, deforestation and loss of biodiversity are major challenges in the process of sustainable global development. In order to fulfill the basic requirements such as food, fuel, medicines and shelter, humans are completely dependent on natural resources. However, continuous increase in global population and associated food

problems have magnified the aforementioned threats and necessitated the sustainable use of natural resources. Food and medicines are integral part of human life and to fulfil the growing demand, continuous global efforts are underway for increasing agricultural productivity. The United Nations Food and Agricultural Organization (FAO) assuming that global population will be about 9.1 billion in 2050 (Godfray *et al.*, 2010). It is reported that 83% medicinal plants have

become endangered mainly due to the human activity (European Commission, 2008; Ibrahim *et al.*, 2013). Whereas, over-utilization of natural resources, pollution of the soil, water and the atmosphere, and introduction of invasive species have resulted into reduced biodiversity (Hunde, 2007).

Medicinal plants are important for the wellbeing of human population and there is an increasing demand for the production of plant-derived secondary metabolites/ novel drug leads (Atanasov *et al.*, 2015). Currently, there is constant demand for plants and plant parts in pharmaceutical industries as well as from Ayurveda professionals. Furthermore, due to major public concerns about dreadful diseases such as cancer, HIV etc., pharmaceutical industries are actively engaged in production of plant-derived drugs. Due to the toxicity and side effects of synthetic drugs, the plant-derived drugs are becoming more popular and as a result there is increase in the number of herbal drug manufacturers (Verma and Singh, 2008; Agrawal, 2005; Lahlou, 2013).

The projected escalating demand for medicinal plants is increasing which leads to unscrupulous collection from the wild and adulteration of raw material supplied to the manufacturers. Ultimately, this practice has resulted into the overharvesting of many plants from wild and disturbed the population of various medicinal plant species and several species even became endangered (Kala *et al.*, 2006; Rao *et al.*, 2004). It has been revealed that more than 50,000 plant species are used in phytotherapy and medicine of which 2/3 are harvested from nature leading to local extinction of many species or degradation of their habitats (Tasheva and Kosturkova, 2012). Due to the constant expansion of herbs trade, the insufficient cultivation fields, and the weak management of harvesting and overharvesting of medicinal plants have led to exhaustion of the natural resources and reduction in the biodiversity. Medicinal plants are always in demand even

though they are facing the threat of becoming endangered and or extinct (Manohar, 2012).

One third of the global plant species are threatened at different level according to International Union of Conservation of Nature (IUCN, 2013). Furthermore, the habitat destruction and loss also leads to the fragmentation of the remaining habitat which eventually results in further isolation of the respective plant species population. The destructive harvest of underground parts of slow reproducing, slow growing and habitat-specific plant species are the crucial factors in making them vulnerable and rare (Ghimire *et al.*, 2005; Kala, 2005). Providing high quality planting material for sustainable use and thereby saving the genetic diversity of plants in the wild is important (Krishnan *et al.*, 2011). However, due to the human intervention there is rapid dwindling of plant resources for medicines; hence, alternative strategies and or innovative approaches are needed for their conservation. Bukuluki *et al.* (2014) had scrutinized the harvesting practices of medicinal plants in Uganda and identified harvesting methods for sustainable supply of medicinal plants. The good harvesting practices suggested include, careful harvesting of roots without affecting tap root, careful removal of stem bark to avoid damaging the innermost layer that contributes to drying of the plant, plucking of leaves without breaking the shoots, picking flowers those are fallen down or selecting only a few in order to allow the plant to bear fruits and reproduce. Recently, Hishe *et al.* (2016) reviewed the value chain of medicinal plants and the associated challenges. They have conducted detailed studies of modes of harvesting, storage, packaging, supply and distribution of medicinal plants. They highlighted that the medicinal plants supply chains have varying requirements for their cultivation, resource management in the wild, harvesting, processing and marketing. Considering these facts, they had concluded that in order to become com-

petitive in the medicinal plants global market place, value chain must become more flexible, innovative and efficient, so it can bring to market new products in a timely fashion.

As medicinal plants represent consistent part of biodiversity, their utilization and conservation strategies needs planned management for sustainability. Therefore, systematic efforts should not only be directed towards preservation of the plant populations but also elevating the level of knowledge for sustainable utilization of these plants in medicine (WHO 2010). Developing strategies for long-term sustainable supply of medicinal plants is challenging; therefore, it has been suggested that to meet future public food and healthcare demand, integration of conventional methods and biotechnology are essential. Biotechnological methods not only offer faster cloning and conservation of the genotype of the plants; but also enable genetic modification, gene regulation and expression for an efficient production of valuable natural substances in higher amounts or with better properties (Tasheva and Kosturkova, 2012). Because of innumerable advantages of biotechnology in agriculture, pharmaceuticals, forestry, food industry and other sectors, the field of biotechnology has become a center of attraction for conservation and sustainable supply of medicinal plants.

2. Biotechnological approaches for conservation of medicinal plants

It appears that biotechnology is emerging dramatically as a key enabling technology for environmental protection and stewardship in a sustainable manner (Cantor, 2000; Gavrilescu, 2004; Arai, 2006). Biotechnological advances have encompassed almost every aspect of human life including food, fuel, cosmetics, medicines and beverages. Most importantly,, biotechnology based-methods are reliable and provides continuous supply of raw material and natural products

for food, pharmaceutical and cosmetic industries (Nalawade *et al.*, 2003). A systematic concept of sustainability was proposed by Prescott-Allen and Prescott-Allen (1996). According to them, both humans and ecosystem are interrelated and dependent on each other. Hence, in conceptual terms, the essence of sustainable development is expressed by the relationship between people and the ecosystem around them. They further stated that the society is thought to be sustainable when both the human condition and the condition of the ecosystem are satisfactory or improving. They concluded that the system improves only when both the condition of the ecosystem and the human condition improve (Prescott-Allen and Prescott-Allen, 1996).

In order to supply medicinal plants or medicinal plant-based raw material in a sustainable manner, various *in situ* and *ex situ* strategies (which includes *in vitro* techniques, botanical gardens, plant banks, GenBank, gene sanctuaries and seed banks) have been suggested for the conservation of critically endangered plant species (Khan *et al.*, 2012). Genetic diversity preservation is of prime importance while conserving plant genetic resources. For the conservation of plant and or their germplasm, *ex situ* and *in situ* strategies are used. The *in situ* approach includes the maintenance of plant species and or their populations in their habitats, where they can naturally occur, grow and reproduce. Whereas, *ex situ* approach of conservation focuses on the maintenance of plant species germplasm under controlled conditions (Pathak and Abido, 2014; Rai *et al.*, 2010). The multiplication of plants by classical methods such as cuttings, budding, layering, and or grafting in nurseries produces enormous number of plants. However, biotechnological methods such as micropropagation, metabolic engineering and genetic manipulations are especially appropriate for species which are difficult to propagate *in vivo* (Tasheva and Kosturkova, 2012). Hence, *in situ* approach of conservation

alone would not be efficient and effective strategy for conservation and multiplication of medicinal plants. Krishnan *et al.* (2011) suggested that prudent application of propagating biotechnology tools in plant conservation program is a prerequisite to succeed (in sustainable use of medicinal plants) and to complement the existing *ex situ* measures. The systematic study on genetic diversity of rare and endangered plant species is very important mainly because, it will be helpful in formulating plans for management and preserving their genetic diversity as well as ensuring their long term survival

(Sreekumar and Renuka, 2006). Biotechnological approaches for sustainable supply and conservation of medicinal plants include micropropagation, mycorrhization, genetic transformation and development of the DNA banks (Sheikhpour *et al.*, 2014; Rai *et al.*, 2010). Figure 1 depicts the biotechnological approaches useful in sustainable supply of medicinal plants. Biotechnological approaches can be used to conserve plants from any group. Table 1 shows some examples of successfully conserved plant species using biotechnology approaches.

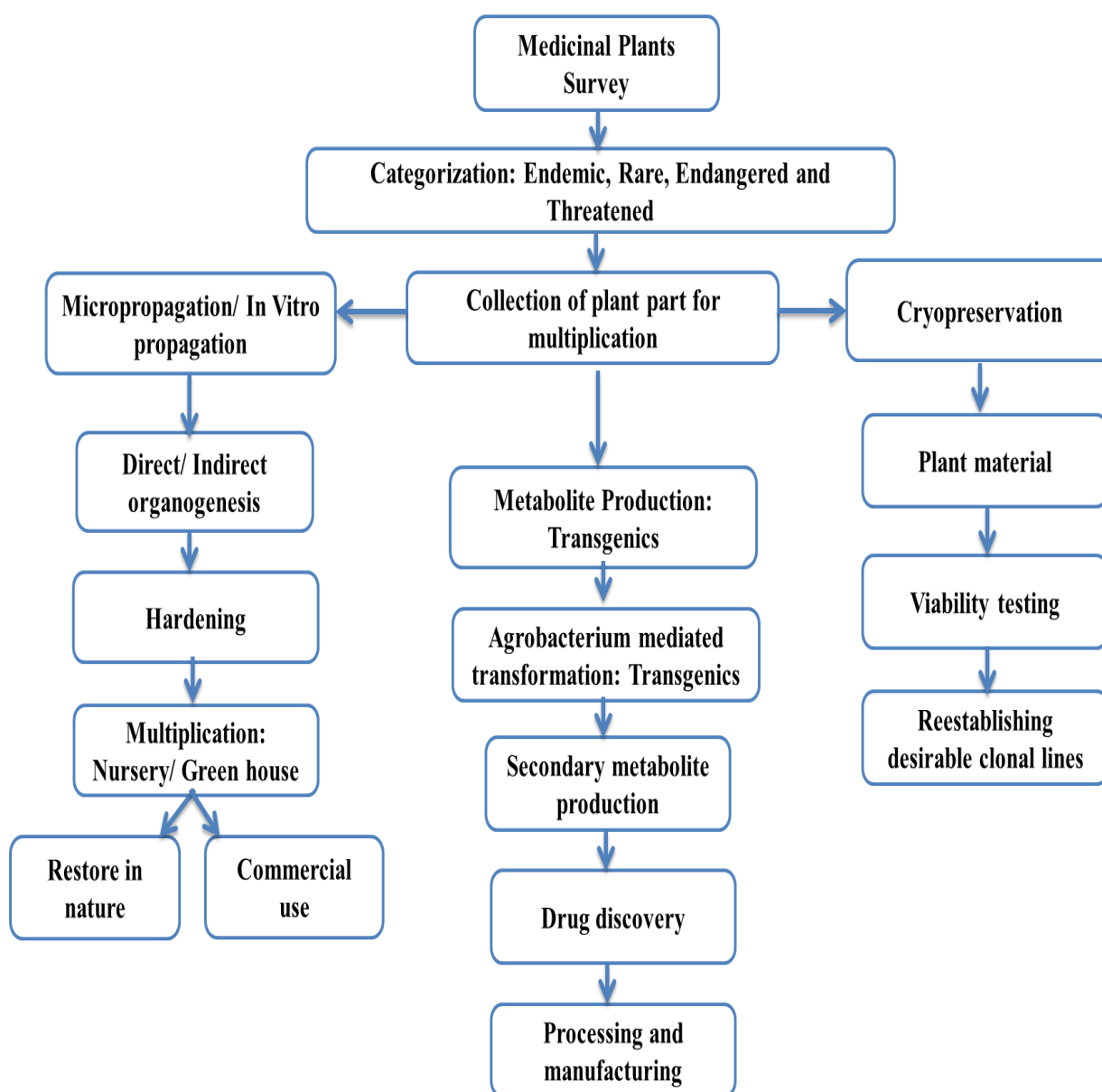


Figure 1: Biotechnological approaches for the sustainable supply of medicinally important plants.

Table 1: Names of some endangered medicinal plants those are propagated using plant tissue culture techniques

Name	Family	Common name	Explant used	Reference
<i>Aerva lanata</i> (L.) Juss. ex Schult.	Amaranthaceae	Chaya	Node	Shekhawat and Revathi, 2017
<i>Bacopa monnieri</i> L.	Scrophulariaceae	Bramhi	Leaves, node & shoot apex	Rathore and Singh, 2013
<i>Commiphora wightii</i> (Ar.) Bhandari	Burseraceae	Guggul	node, shoot tip, & axillary bud	Tejovathi <i>et al.</i> , 2011
<i>Gentiana kurroo</i> L.	Gentianaceae	Indian gentian	Node	Verma <i>et al.</i> , 2012
<i>Paris polyphylla</i> sm.	Trilliaceae		Bulb	Verma <i>et al.</i> , 2012
<i>Picrorhiza kurroa</i> Royle ex. Benth	Scrophulariaceae	Kaur	Nodal sector	Jan <i>et al.</i> , 2010
<i>Picrorrhiza kurroa</i> Royle ex Benth.	Scrophulariaceae	Satuva	Leaf/ node	Verma <i>et al.</i> , 2012
<i>Psoralea corylifolia</i> Linn	Fabaceae	Indian bread root	Apical meristem	Pandey <i>et al.</i> , 2013
<i>Psoralea corylifolia</i> Linn	Fabaceae	Kutki	cotyledons, hypo-cotyls	Sehrawat <i>et al.</i> , 2013
<i>Rheum emodii</i> L.	Polygonaceae	Himalayan rhubarb	Basal disc	Verma <i>et al.</i> , 2012
<i>Salvia sclarea</i> L.	Labiataeae	Clary sage	Node	Verma <i>et al.</i> , 2012
<i>Saussurea esthonica</i> Baer ex Rupr.	Asteraceae	--	Seeds	Gailite <i>et al.</i> , 2010
<i>Stevia rebaudiana</i> (Bertoni) Bertoni	Asteraceae	Candy leaf	Leaf/Node	Verma <i>et al.</i> , 2012

Table 2: Some examples of successfully conserved plant species using biotechnology approaches

Plant species	Approach	Explant used	Reference
<i>Calophyllum apetalum</i>	Micropropagation		Lakshmi and Seeni, 2003
<i>Cineraria maritima</i> L.	Cryopreservation-Encapsulation	Shoot tips and nodal segments	Srivastava <i>et al.</i> , 2009
<i>Chlorophytum borivilianum</i> Sant. Et Fernand	Micropropagation	Floral buds	Sharma and Mohan, 2006
<i>Decalepis arayalpathra</i>	Micropropagation		Gangaprasad <i>et al.</i> , 2005
<i>Dioscorea floribunda</i>	Cryopreservation	Shoot tip	Ahuja <i>et al.</i> , 2002
<i>Gomortega keule</i> (Mol.) Baillon	Micropropagation	Zygotic embryos	Muñoz-Concha and Dave, 2011
<i>Psoralea corylifolia</i> L.	Micropropagation	Apical meristem	Pandey <i>et al.</i> , 2013

2.1. *In vitro* culture and micropropagation

Plant tissue culture is the *in vitro* culture of plant cells, tissues, and or organs in sterile nutritionally and environmentally controlled conditions. In micropropagation, *in vitro* multiplication of large number of plants from explants such as leaves, seeds, nodes, anthers, ovary and tubers etc. is carried out. The plantlets thus propagated can be used for production and multiplication of plantlets which are genetically similar. In addition to the conservation of rare and endangered medicinal plants, micropropagation is considered as the oldest commercial biotechnology based clonal plant propagation method (Rai *et al.*, 2010). Development of reliable *in vitro* culture protocols is of great importance for conservation of rare and endangered medicinal plants. So far number of *in vitro* protocols has been developed for rare/endangered medicinal plants to propagate them at large scale (Shahzad and Saeed, 2013). Table 2 shows some examples of successfully propagated rare/endangered medicinally important plants using micropropagation technique. An efficient micropropagation system for endangered Chinese medicinal plant, *Saussurea involucrata* has been developed from leaf explants. Similarly, *In vitro* culture of *Saussurea esthonica*, an endangered wild plant species in Latvia was performed using seeds (Gailīte *et al.*, 2010). Tropical Botanic Garden and Research Institute, India has developed *in vitro* protocol for rapid regeneration and establishment of about 40 medicinally important rare and threatened plants of Western Ghats. Similar attempts of medicinal plants conservation were made by Verma *et al.* (2012). Their studies included *in vitro* conservation of 23 over-exploited medicinal plants that belonging to the Indian Sub-Continent (Verma *et al.*, 2012). It is important to note that Synthetic seeds were also produced from highly proliferating shoot cultures of some plants. Out of 23 plants, 18 plants were

successfully hardened under glasshouse conditions.

Seed encapsulation techniques are considered as very promising for conservation purposes as the protection provided to the plant material by encapsulation could increase its resistance to dehydration and low temperature, thus opening new possibilities for medium-term storage (Ray and Bhattacharya, 2010). These studies clearly suggest that the efficient *in vitro* propagation system developed for rare, endangered and or economically important medicinal plants are very useful in their conservation and supply for a sustainable growth and development of the industry.

2.2. Metabolite Engineering and Genetic Manipulations

Plant-derived secondary metabolites are in great demand and researchers are actively engaged in secondary metabolite production using various *in vitro* techniques. For instance, transgenic for overexpression of gene/s in secondary metabolite pathway have not only provided alternative but also offer sustainable approaches towards conservation of medicinally important plants. Genetic transformation or transgenic technology (also referred as GM technology) have been successfully resulted in adjunct to classical plant breeding, in that it allows the targeted manipulation of specific characters using genes from a range of sources (Shewry *et al.*, 2008). *Agrobacterium* mediated plant transformation approach was successful in a number of non-food and food crops mainly due to its simplicity and efficiency; but, it is still not used widely to improve the quality of medicinal plants (Tashdeva and Kosturkova, 2012). One of the most appropriate methods for medicinal plants engineering is genetic transformation using *Agrobacterium rhizogenes* leading to increased synthesis of secondary metabolites in root cultures or in regenerated plantlets. For instance, studies on hairy root cultures

using *Agrobacterium rhizogenes* suggested that the intact plant synthesizes a large quantity of biologically active substances and therefore, their production could be increased significantly by transformed roots cultures as well as by transformed plants (Wang *et al.*, 2015; Tashdeva and Kosturkova, 2012; Georgiev *et al.*, 2007; Guillon *et al.*, 2006). For example, *Psammosilene tunicoides* is a medicinal herb endemic to China and due to excessive destructive exploration; natural resources of this plant species have dwindled and species become threatened (Wang *et al.*, 2015). Considering its medicinal importance, successful hairy root culture has been established using genetic transformation of plant tissues by *Agrobacterium rhizogenes* aiming to enhance the secondary metabolites production (Wang *et al.*, 2015). As the continuous harvesting of medicinal plants lead to the exhaustion, use of *Agrobacterium rhizogenes* to transform medicinal plants for secondary metabolite production under laboratory conditions would not only provide the protection and conservation of rare or endangered medicinal plant species but also offers a sustainable approach.

The sustainable plant production is also possible by using microbial inoculants as substitution for chemical fertilizers and pesticides (O'Gara, 1996). Inoculation of mycorrhizal fungi into the roots of plants is referred as mycorrhization (Williams *et al.*, 1994). This can be achieved by delivering microbial inoculants via micropropagation (Dolcet-Sanjuan *et al.*, 1996). Several reports suggest that inoculation of arbuscular mycorrhizal fungi (AMF) into the roots of micropropagated plantlets plays an advantageous role (Sylvia *et al.*, 2003; Voets *et al.*, 2005; Chandra *et al.*, 2010).

Tools such as *in-vitro* propagation, mycorrhization and genetic engineering not only hold tremendous potential to select, multiply and conserve the critical genotypes of medicinal plants but also offer the production of high-quality

plant-based medicine (Sheikhpour *et al.*, 2014; Tripathi and Tripathi, 2003). As plant-derived drugs have lesser side effects in comparison to allopathic medicine, medicinal plant species have made an outstanding contribution in many traditional herbal therapies practiced in various parts of the world. Considering the importance of medicinal plants, efforts should be made at different levels for their sustainable supply (Kala *et al.*, 2006). Due to accelerated local, national and international interest, the demand for medicinal and aromatic plants has grown rapidly and therefore, public–private collaboration is essential (Van De Kp *et al.*, 2006).

2.3. Cryopreservation

One of the important biotechnological tools in conservation of plant species is cryopreservation which includes freeze- preservation or cryogenic storage of biological material at a very low temperature (Jain *et al.*, 2012). Cryopreservation approach is used to conserve plant germplasm when other traditional approaches such as seed banking and vegetative propagation do not work efficiently for the respective plant species. Hence, for long-term conservation of *in vitro*-derived plant germplasm is stored in liquid nitrogen (-196°C) (Engelmann, 2011). It has been reported that, as threatened and endangered species produce little or no viable seeds or are dormant; hence, the preservation of remaining individuals is considered of paramount importance (Bunn *et al.*, 2007) and such problematic species needs to be maintained through cryo-collections (Kaczmarczyk *et al.*, 2012). Cryopreservation ensures safe and cost-efficient long-term conservation of species without the loss of viability, so when required, material can be readily retrieved and reinitiated, reestablishing desirable clonal lines (Shibli *et al.*, 2004). Plant materials such as cells, tissues, gametes, oocytes, organs, DNA samples etc. are stored so that they can be used in future (Sharma and Sharma, 2013). Cryo-

preservation is considered as one of the most important conservation techniques and has been a successfully approach for the conservation of number of medicinal plants. Conservation of *Atropa belladonna*, *Digitalis lanata*, *Hyoscyamus sp.* and *Rauvolfia serpentine* are some examples to state (Jain *et al.*, 2012). Initiatives for conservation of medicinally important endangered plants have already been taken by several institutions. For instance, more than 110 accessions of rare or threatened plant species are stored using cryopreservation approach of conservation at the Kings Park and Botanic Garden in Perth, Australia (Touchell and Dixon, 1994). Likewise, The National Bureau for Plant Genetic Resources (NBPGR, New Delhi, India) has stored more than 1,200 accessions from 50 different plant species (Mandal, 2000). Shoot tips of endangered and medicinally important plant, *Picrorhiza kurroa* have been preserved using same approach (Sharma and Sharma, 2003). Cryo-preserved embryogenic cultures of *Dioscorea bulbifera* was performed using an encapsulation-dehydration procedure. After cryopreservation, the sub-culturing showed 53.3% recovery of growth of embryogenic culture (Mandal *et al.*, 2009). These studies clearly provide the insights about the usefulness of cryo-techniques in conservation of valuable medicinal plants (Tashdeva and Kosturkova, 2012). However, research on cryopreservation techniques is relatively limited; hence, further research and development is essential in this area to utilize this technique/approach efficiently for the conservation of medicinal plants.

3. Perspectives

Understanding of the challenges and opportunities in conservation of medicinal plants and to develop the biotechnological approaches to deal with it is important for the sustainable supply of medicinal plants. Medicinally important plants are continuously harvested for their

traditional applications and for the development of novel drugs and or supplementary products. However, plant-derived drugs as medicines have been based on the assumption that the plants will be available on a continuing basis. As much more attention is given for discovery of new drugs from medicinal plants, the demand for raw material is steadily growing. However, there are no enough meticulous efforts to ensure the availability of targeted medicinal plants for harvesting to fulfil the industry's demand. Moreover, there are no sustainable approaches developed for harvesting as well as for conservation of in-demand medicinal plants. Due to this awful situation, many medicinal plant species are becoming rare, vulnerable, endangered and or extinct. Unfortunately, very meager efforts have been undertaken for their conservation and sustainable supply. Therefore, there is a need to intensify the efforts for not only for the conservation but also to ensure sustainable supply of medicinal plants. Biotechnological tools such as micropropagation, cryopreservation and transgenic approach should be used efficiently for the conservation of medicinal plants for the sustainable growth and development of the industry, people and the planet.

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Making Himalayas Sustainable: Opportunities and Challenges in Indian Himalayan Region

Harsh Kumar Chauhan and Anil Kumar Bisht*

Department of Botany, D.S.B. Campus, Kumaun University, Nainital, 263001, Uttarakhand, India; *Correspondence: bishtakb@rediffmail.com; Tel: +91 9412044500

Abstract: Himalayas are among the most vulnerable ecosystems of the world. They harbor unique geology/geography, ecosystem, biodiversity, and several other life sustaining biotic and abiotic resources. Sustainability of Himalayas is being challenged by increased tourism activities, deforestation, pollution, unmanaged exploitation of bio-resources, climate change and unplanned developmental activities. Besides, the region has several resources of aesthetic and economic interest that can be harnessed for generation of income and employment to millions of the people residing in the region. Understanding the specific problems of Himalaya and carving out the prospects for its sustainable development is a difficult task. Looking at the present scenario, the chapter provides an overview of the opportunities and challenges for achieving sustainability in Indian Himalayan Region. The present trends suggest that the existing interventions in the region are unsustainable. Further, the unscientific exploitation of natural resources is increasing the environmental degradation in the region. Proper policies and their implementation for harnessing the potential of the natural resources are urgently needed for the sustainable development of the region.

Keywords: Himalayas; hotspot; sustainability; unscientific exploitation; vulnerable

1. Introduction

The Himalayas are among the most vulnerable mountain ecosystem stretching between the Indus and Brahmaputra river valleys (Bawa *et al.*, 2010). They spread across the eight countries; Afghanistan, Bangladesh, Bhutan, China, India, Myanmar, Nepal and Pakistan. The Himalayas harbor unique biodiversity, ecosystem composition and several other life sustaining resources. They are the source of 10 of the largest rivers in Asia which provides water to about 1.3 billion people (Xu *et al.*, 2007; Bates *et al.*, 2008). In addition to provide water, the Himalayas provide huge inputs to agriculture through regulating micro-climates as well as wind and monsoon circulation (Rasul, 2010) supporting life of about 40 million people (Zurik and Pacheco, 2006). They are known to facilitate vital ecologi-

cal and economic security of the people living downstream. They are also considered as the repository of geological and agriculture assets and harvested wild goods (Badola *et al.*, 2015).

Unfortunately, the entire region is prone to several disasters due to fragile geophysical structures, high peaks, and high angle of slope and variable climatic conditions (Chhetri, 2001). This vulnerability increases several folds with the increasing human population, exploitation of the natural resources and the effects of the climate change (Liu and Chen, 2000; Dyurgerov and Meier, 2005). Poverty in the Himalayan region is high and persistent (Hunzai *et al.*, 2011). Some of the areas in the region are under territory disputes between the nations which are associated with the military presence along the international border; for instant the degradation of the Hind Kush, Karako-

rum, Western Himalaya and the Kashmir. This situation is particularly damaging to fragile ecosystem whose recovery is particularly slow (Bawa *et al.*, 2010). The political situation thus prevail prevent the proper policy implementation for developmental programs causing the unfair treatment of the people residing in these areas. These synergistic effects seem to make Himalayas the hotspot for the physical, economic and social vulnerability.

The studies carried out on the mountain ecosystems throughout the world concluded that mountains are in dire need of relief from anthropogenic activities (Jodha, 2005). Sustainability of Himalayas is being challenged by increased tourism activities, deforestation, pollution, climate change and unplanned development. Besides all this, the region has several resources of symbolic and economic values whose harnessing can provide income and employment to millions of the people residing in the region. Understanding the specific Himalayan problems and prospects of the sustainable development is not an easy task. International failure to recognize the economic value of the issues of sustainability using policy tools in the Himalayas has been cited as the major cause of this continued

degradation (Singh, 2002; Blaikie and Muldavin, 2004). Several scientific and political forums have emphasized the uniqueness, environmental challenges and political legacies of the Himalayan region so that sustainable planning and management in the region can be worked out. Global change and World's Mountains conference held at Perth, Scotland in 2010 also identified several research gaps in sustainable mountain development. Looking at the current scenario, the present chapter provides the overview of the opportunities and challenges of sustainability in the Himalayas with special reference to Indian Himalayan Region.

2. Indian Himalayan region (IHR)

IHR occupies a special place in the mountain ecosystem of the world (Singh, 2006). The region extends between latitude 26°20' and 35°40' North, and between longitudes 74°50' and 95°40' East covering 530, 795 sq. km of geographic area. It spreads across the states of Jammu and Kashmir, Himachal Pradesh, Uttarakhand, Sikkim, Arunachal Pradesh, Meghalaya, Nagaland, Manipur, Mizoram, Tripura and the hill regions of Assam and West Bengal (Figure 1). It co-

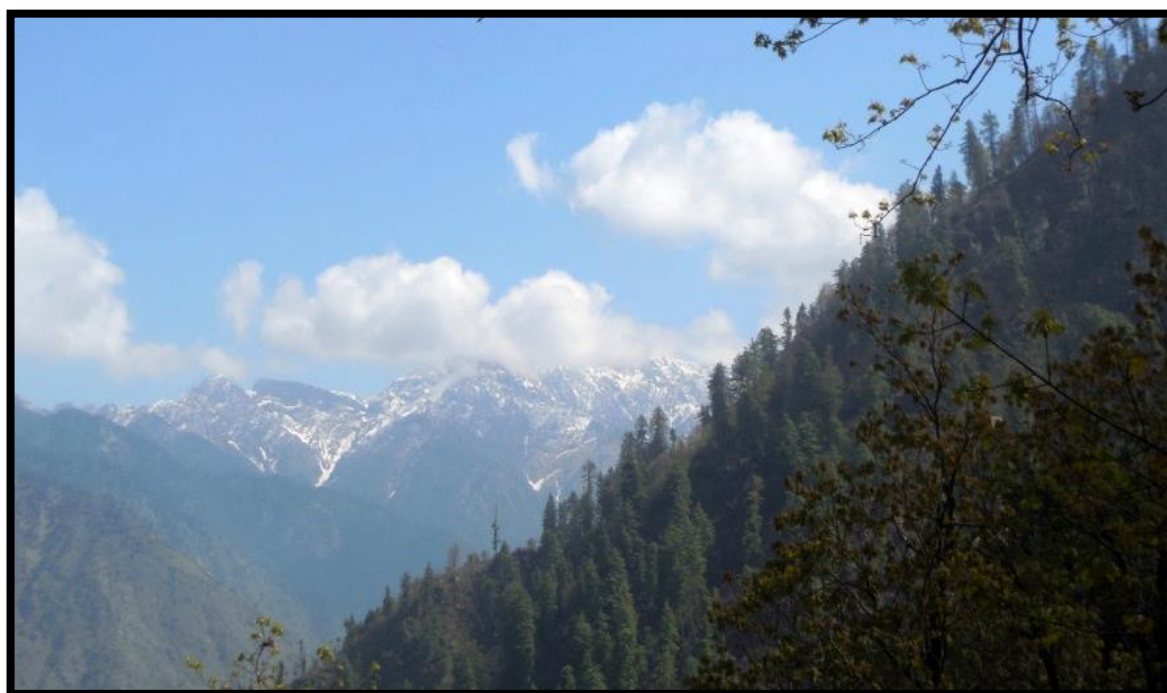


Figure 1: A picture of the Himalayan landscape.

nstitutes about 16.2% of India's administered geographical area. Most of the area in the region is covered with the snow clad mountain peaks, glaciers in the higher Himalayas and dense forest in the mid-Himalayas thus harbouring a rich variety of flora, fauna and cultural diversity.

3. Opportunities and challenges for sustainability in the IHR

3.1. Water potential

Himalayas has been rightly acknowledged as the 'Water Tower of Asia'. Approximately 10-20 % of the area is covered by glaciers while 30-40% remains under seasonal snow cover (Bahadur, 2004). Being the source of Asia's 10 largest rivers (Amu Darya, Indus, Ganges, Brahmaputra, Irrawaddy, Salween, Mekong, Yangtze, Yellow and Tarim) (Xu *et al.*, 2007) they provide drinking water, irrigation, fisheries, hydropower, and supports several terrestrial and aquatic ecosystems. The Ganges River system is the main source of fresh water to more than half the population of India and Bangladesh and nearly entire population of Nepal (Rasul, 2014). About 60% of the India's irrigated area of 546,820 Km² is in the Ganges basin (National Ganga River Basin Authority, 2011). Indus irrigation system irrigates about 14.3 million hectares of farmland constituting the world's largest contiguous irrigation system; enabling the production of more than 80% food grains of Pakistan (GoP, 2010). Amu Darya irrigates 385,000 ha of farmland in Afghanistan contributing a reliable source of Afghanistan's food and water security (NAS, 2012). The ground water flow through bedrocks is approximately six times the annual contribution from glacial ice melt and snow melt to central Himalayan Rivers (Andermann *et al.*, 2012).

The government of India had recognized the hydropower potential of the IHR. The country's hydropower potential is 148,701 MW out of which more than 75% (117,139 MW) resides in IHR; however only 22.37 % potential has been de-

veloped and 9.09% is under construction (CEA, 2009). Accordingly the Prime Minister of India launched a 50,000 MW hydroelectric initiative program, formulated by CEA for preparation of Preliminary Feasibility reports of 162 new hydropower schemes (47,930MW) and out of these 133 are in IHR (Agarwal *et al.*, 2010).

The development of hydropower offers several advantages to the economy of the nation. It is the source of clean renewable source of energy offering the mitigation of climate change issues and achieving the sustainability goals. It provides the inexpensive power, especially when the project achieves financial breakeven. The development of the hydropower project is expected to improve the infrastructure of the remote areas as well as it helps in flood moderation, irrigation, navigation and providing drinking water all the year around.

Unfortunately several challenges are associated with the development of the hydropower in IHR. It has been realized that the development of the hydropower projects has significant environmental and social impact (Goldsmith and Hildyard, 1984). These projects alter the vital ecological process such as flow of water, sediments, nutrients, energy and biota (Franklin *et al.*, 1995). The IHR is among the most seismically active zone of the world; the construction of the hydropower project increases the chances of the earthquakes which may shatter the lives of millions of people. Proper Environment Impact Assessment appears to be the biggest challenge for the implementation of the hydropower project in IHR. In a nutshell it may cause multi-dimensional unpredictable ecological disturbances and loss of biodiversity, productive land, social and cultural heritage. Hence, the development interventions related to hydropower in the IHR should have different approach. As per the reports of Chopra committee, formulated by the order of Supreme Court of India in the year 2014 to assess the role of hydroelectric projects

in Uttarakhand, the negative impacts of the small hydropower projects can be less intense and therefore mitigated more easily. On the other hand, large projects often lead to massive impacts that are hard to mitigate and may result in permanent scarring of nature and society. Further, the apex court also warned of negative impacts on geological environment, river ecosystem & forests and terrestrial biodiversity (Table 1).

3.2. Biodiversity

Climatic, topographic, geological and altitudinal variations have generated unique landscape (Figure 2), ecosystems

and biodiversity in the Himalayas (Shrestha *et al.*, 2012). The complex orogeny of the Himalayas, coupled with climatic and edaphic changes facilitate the colonization of floral and faunal diversity in the region (Pandit *et al.*, 2000). Himalayas are the repository of the extremely rich and endemic biodiversity (Chatterjee, 1939; Nayar, 1996; Pandit *et al.*, 2000). The region hosts the parts of four global biodiversity hotspots (viz. the Himalayas hotspot, the Indo-Burma hotspot, South-West China Hotspot and the Mountains of Central Asia hotspot) (Mittermeier *et al.*, 2004). Himalayas are very important in terms of sustaining high levels of the

Table 1: Negative impact of hydropower projects*

Activity	Impact
I. Pre-project construction	
1. Construction of approach roads	• Land acquisition (displacement, loss of lands, homes and livelihoods) • Deforestation (loss of tree cover, access to CPRs, soil erosion and landslides, loss of flora and fauna, changes in micro-climate) • Disposal of debris and earth (loss of trees, river water pollution)
2. Construction of housing for staff and labour	• Deforestation • Pollution due to sewage release
3. Quarrying	• Noise pollution, slope destabilization, disruption of underground seepage and damage to house.
II. Project construction	
4. Tunneling	Air and soil pollution, destabilization of slopes, damage to houses, disturbing wildlife, drying of springs, disposal of muck into the river, psychological trauma to people and animals due to repeated blasts
5. Dam construction	Disruption of river flows (biotic changes, disruption of natural functions, e.g. sediment disposal, land shaping, nutrient cycling), river pollution, loss of aesthetic, cultural, economic and recreational values.
III. Project operation	
6. Testing of tunnels	Slope destabilization (loss of tree cover, land, livelihoods, water sources and access to CPRs)
7. Water storage and release	• Sedimentation (effect on river water quality) • Disruption of river flow • Secondary effects (release of greenhouse gases, warming of valleys, increased earthquake risks, floods, downstream urban and industrial development)
8. Laying of power lines	Deforestation (loss of wild life habitat), soil erosion

*Information source: <http://iced.cag.gov.in>

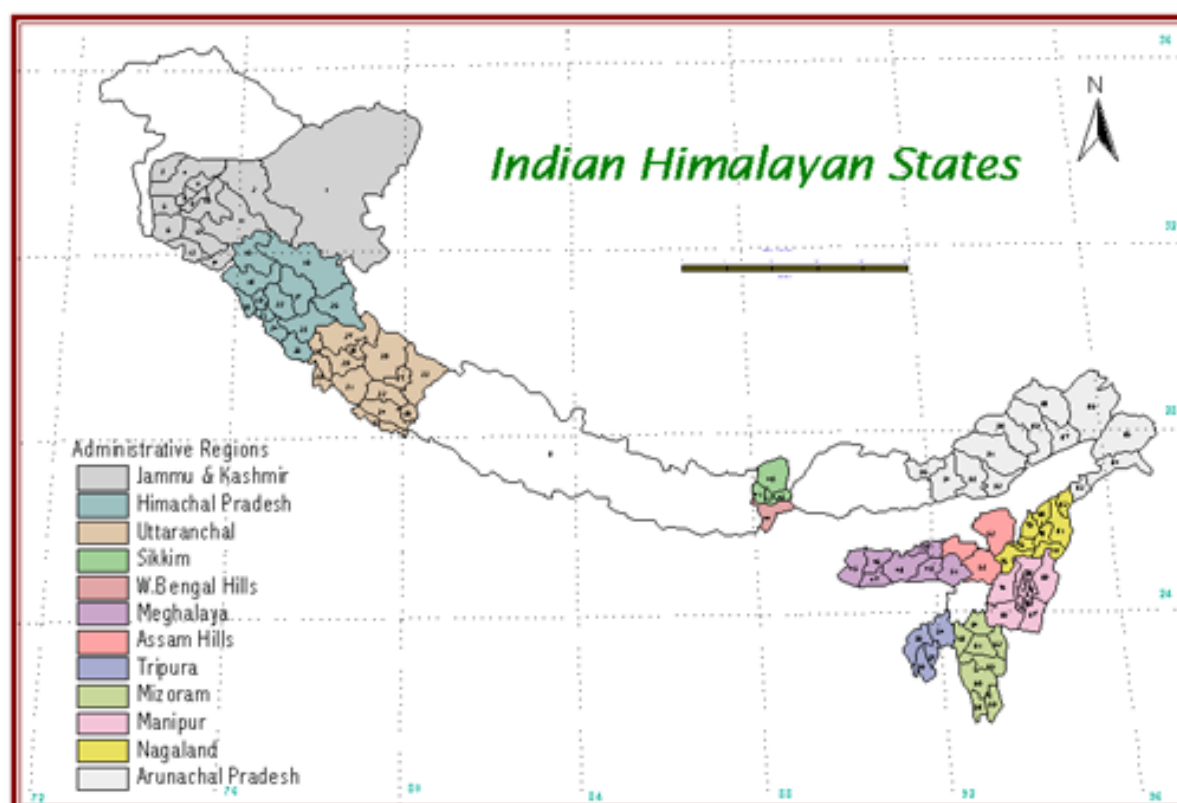


Figure 2: Indian Himalayan Region (IHR) (Source: [www.http://gbpihedenviis.nic.in/indian_him_reg.htm](http://gbpihedenviis.nic.in/indian_him_reg.htm))

biodiversity. However, biodiversity loss from the region had become a matter of great concern. There are several factors such as anthropogenic pressure and the climate change which contributes to the loss of biodiversity in the region.

Due to the collation of several geographic and climatic features IHR provides very suitable environment for flourishing the huge biodiversity. IHR supports nearly 50% of the total flowering plants in India of which 30% are endemic to the region (Singh *et al.*, 2006). It harbours 816 tree species, 675 edibles and 1748 species of medicinal value (Samant *et al.*, 1998). The forests of the region have phenomenal diversity that meets the diverse needs of the people (Singh and Singh, 1992). The forest of the region acts as the sink of the carbon dioxide and provides timber, wild edibles, gums, resins and other numerous products of immense value.

Massive deforestation, extensive shifting cultivation and the demands for agricultural land are the primary drivers

for the biodiversity loss in IHR. At the current rate of deforestation in IHR, the total forest cover (84.9 % in 2000) and coverage of dense forest (75.4% in 2000) is expected to be reduced to 52.8% and 34% respectively by the year 2100 (Pandit *et al.* 2007). This could have serious implications on the diversity of the flora and fauna of the region. Looking at the ever-increasing threats to the biological diversity in the region; there is an urgent need of the proper actions or otherwise it may bring huge setback to the economic benefits of the local populations.

3.3. Medicinal plants

Among the biodiversity elements, the roles of medicinal plants is remarkable in the health care of the Himalayan people as most of the them resides in the remote locations where the allopathic system of medicines is not practiced to such an extent. Besides the health care, medicinal plants have high socio-cultural, symbolic and economic value, providing income and employment to millions of peo-

ple living in the region (Ghimire, 2008). Himalayan medicinal plants meet most of the international demand. Over-exploitation seems the biggest challenge for the survival of these plants though they also have slow growth rates, low population densities and narrow geographical ranges (Kala *et al.*, 2006).

IHR host 1748 plants species of medicinal value including 1685 angiosperms, 12 gymnosperms and 51 pteridophytes (Samant *et al.*, 1998). Ved *et al.*

(2001) estimated 3200 medicinal plants from Indian Himalayas including 700 from trans-Himalaya (Table 2). India is ranked 2nd after China in terms of medicinal plants export and exports about 32,600 tons of medicinal raw material worth about US \$46 million annually (Lange, 1997). High nativity and endemism of medicinal plants is associated with the IHR. Out of 1748 medicinal plants, about 548 species are identified in HP, 707 in Sikkim and Darjeeling and

Table 2: Medicinal plants, species diversity and representative species of different biogeographic zones of India[#]

Biogeographic region	re-	Estimated no. of medicinal plants	Examples of some typical medicinal species
Trans-Himalayas		700	<i>Ephedra geradiana</i> Wall., <i>Hippophae rhamnoides</i> L., <i>Arnebia euchroma</i> (Royle) John
Himalayan		2500	<i>Aconitum heterophyllum</i> Wall. ex Royle, <i>Ferula jaeshkeana</i> Vatke and <i>Saussurea costus</i> (Balc). Lipsch., <i>Nardostachys grandiflora</i> D.C. <i>Taxus wallichiana</i> Zucc., <i>Rhododendron anthopogon</i> D.Dun and <i>Ponax pseudoginseng</i> Wall.
Desert		500	<i>Convolvulus microphyllus</i> Seib ex Spreng., <i>Tecomella undulata</i> (Sm.) Seem., <i>Citrus colocynthis</i> (L.), Schrader and <i>Cressa cretica</i> L.
Semi-Arid		1000	<i>Commiphora wightii</i> (Arn.) Bhandari, <i>Caesalpinia bonduc</i> (L.) Roxb, <i>Balanites aegyptiaca</i> (L.), Delilie and <i>Tribulus rajasthanensis</i> Bhandari & Sharma.
Western Ghats		2000	<i>Myristica malabarica</i> Lam., <i>Garcinia indica</i> (Thou.) Choisy, <i>Utleria salicifolia</i> Bedd and <i>Vateria indica</i> L.
Deccan Peninsula		3000	<i>Pterocarpus santalinus</i> L.f., <i>Decalepis hamiltonii</i> Wigh & Arn, <i>Terminalia pallida</i> Brandis and <i>Shorea tumbergaia</i> Roxb
Gangetic Plain		1000	<i>Holarrhena pubescens</i> (Buch-Ham.) Wall. ex DC., <i>Mallotus philippensis</i> (Lam.) Muell – Arg., <i>Pluchea lanceolata</i> C.B. Clarke and <i>Peganum harmala</i> L.
North-East India		2000	<i>Aquilaria malaccensis</i> Lam., <i>Smilax glabra</i> Roxb., <i>Ambroma augusta</i> (L.) L.f. and <i>Hydnocarpus hurzii</i> (King) Warb.
Islands		1000	<i>Claophyllum inophyllum</i> L. <i>Adnanthera pavonina</i> L., <i>Barringtonia asiatica</i> (L.), Kurz and <i>Aisandra butyracea</i> (Roxb.), Baehni.
Coasts		500	<i>Rhizophora mucronata</i> Lam., <i>Acanthus ilicifolius</i> L., <i>Avicennia marina</i> Vierth and <i>Sonneratia caseolaris</i> (L.) engl.

[#]Source: Ved *et al.*, 2001; <http://www.fao.org/docrep/007/ad871e/ad871e09.htm>

701 in the Uttarakhand (Badola and Aitken, 2003). The economic potential of the medicinal plants has been recognized in the IHR. It possesses various forest types with diverse habitats, slopes, aspects and altitudinal ranges which offers congenial environment for the natural and artificial propagation of the diverse medicinal plants. Such diversity of the medicinal plants would be helpful for further scientific research on exploring their medical efficacy (Kala *et al.*, 2006). The aim set by India for establishing golden triangle between traditional medicine, modern medicine and modern science will be a boon for development of traditional herbal medicine and medicinal plant sector (Mashelkar *et al.*, 2005).

However, there is a paucity of research on the biology, habitat and adaptation mechanism of the majority of the threatened trade taxa of the medicinal plants (Dhar *et al.*, 2000). Further, proper agro techniques for the cultivation of the medicinal plant are lacking in the Himalayas. It is noteworthy to mention here that < 90% of the plant raw material for herbal and industries in India and for export is drawn from natural habitats (Tandon, 1996, Ved *et al.*, 1998; Dhar *et al.*, 2000). Several challenges are associated with the sustainable development of the medicinal plant sector in IHR. Most important among these are low population size, habitat specificity, narrow distribution ranges, unscientific collection for commercial purposes, land use disturbances, introduction of non-native species, habitat loss and alteration, climate changes, heavy livestock grazing, unregulated tourism, construction of dams and roads, explosion of human population, population bottlenecks and genetic drift (Kala, 2005).

Recent advancement in the field of Plant Biotechnology especially Plant tissue culture has emerged as the promising technique for the conservation of these medicinal plants. *In-vitro* propagation of plants holds tremendous potential for the production of high-quality plant-based

medicines (Fuentes *et al.*, 1993). Using *in vitro* propagation methods several rare and endangered plant species can be quickly and successfully propagated with low impact on wild population (Cuenca *et al.*, 1999). Plant cell cultures represent a potential source of valuable secondary metabolites which can be used as food additives, nutraceuticals, and pharmaceuticals. The major advantage of the synthesis of phytochemicals by the cell cultures is that they are independent of environmental conditions and quality fluctuations. Therefore, this sector undoubtedly offers several opportunities for the sustainable development of the region however there are lots of challenges associated with the sound development of the sector in the region.

3.4. Agriculture assets and food security

Agriculture assets and food security is a critical issue of Himalayas due to complete dependency on rain besides general characteristics of remoteness, low market integration and underdeveloped agrarian resources. However, agriculture and allied sector forms the pivotal part of the people living in the Himalayas. The huge diversity in the Himalayas has been maintained through a variety of crop composition, indigenous methods of maintaining soil fertility, socio-cultural and religious rituals (Negi and Maikhuri, 2013).

IHR is the storehouse for the diverse genetic stocks. For instance, farmers of the Central Himalaya grow about 100 varieties of paddy, 170 varieties of kidney beans, 8 varieties of wheat, 4 varieties of barley and about a dozen varieties of pulses and oil seeds each year and farmers of Uttarakhand Himalaya are known for cultivating 34 crop species comprising of 6 types of cereals, 5 types of pseudo cereals, 6 types of millets, 16 types of pulses, 4 types of oilseeds, 5 types of condiments and 8 types of vegetables (Negi and Maikhuri, 2013). The wild fruits of IHR have significantly attracted the attention of the entire world from the Nutraceutical

point of view. However, at present the decline in the interest of farming has been observed as a result of climatic uncertainty and cultural transformation. The consequences of this may be disastrous because of genetic erosion of some unique and diverse gene bank.

3.5. *Tourism /aesthetic/ ecological services*

Tourism industry typically depends on the quality of the natural resources. Himalayas are blessed with natural beauty and pilgrim centers which attracts the millions of International tourists throughout the year. They have abundant of natural resources like spectacular landscapes, mountains, glaciers, rivers, lakes, fountains, snow clad peaks, forests having high floral and faunal diversity, which offers strong resonance with tourism. These bioresources make Himalayas suitable for establishing Sanatoria and rejuvenating centers. Thus, they have huge potential for promoting tourism in the form of natural and cultural heritage.

Economic potential of tourism that could promote to sustainability in IHR is well recognized. IHR host several tourist destinations which provides livelihood to millions of people residing in the region. With the arrival of Britishers in 19th century, several hill stations like Nainital, Darjelling, Mussoorie, Shimla etc. were established. They are the tourist hotspots for thousands of national and international tourist. In India, both the state and central government have declared tourism to be an industry and provides same concessions and incentivizes of the industrial sector (Cole and Sinclair, 2002).

However, several challenges are associated with the sustainable development of the tourism industry in IHR. Hollen, (2010) had identified lack of confidence in the economic certainty of tourism as the major challenge in the sustainable development of the tourism in Himalayas. He also advocates the philosophy of sustainable development constructed upon conservation, community participa-

tion and social equality. In order to utilize the tourism industry market, uncontrolled number of tourist there should be proper infrastructural facilities. The ecological pressures are threatening land, water and wildlife resources through direct and indirect environmental impacts together with generation of solid and liquid wastes (Singh, 2002). So, ecotourism or green tourism should be promoted for the sustainable development of the region.

3.6. *Other challenges (disasters, climate change, population increase, poverty and waste management)*

People living in IHR, are facing the problems and damages due to some sudden events such as forest fire, avalanches, cloud bursting, land and mudslides, earthquakes, debris flows, flash floods, paralyzes the life and property of the people (Nyaupane and Chhetri, 2009). There are several evidences of Climate change and its impact in Himalayas (Beniston, 2003; Cruz *et al.*, 2007; Xu *et al.*, 2009). Among these impacts, the most widely reported is the receding of glaciers which could have disastrous impact on the survival of the millions of people. The ongoing climate change over succeeding decades will likely to have additional negative impacts across these mountains, including significant cascading effects on the river flows, ground water recharge, natural hazards, and biodiversity; ecosystem composition, structure and human livelihoods (Xu *et al.*, 2009). The population in Himalayas is rapidly increasing; however there are finite resources to enhance the production ultimately promoting the poverty. Recently proper waste management appears as the critical problem in the Himalayas. Reducing forest cover, accelerated soil erosion, drying springs, biodiversity loss etc. seems the ever increasing challenges for the sustainability in the Himalayas.

4. **Concluding remarks**

Himalayas have undoubtedly huge ecological, life sustaining, recreational, educational and scientific values. Several challenges are associated for the sustainability in the region. The present trends suggest that the existing interventions in the region are unsustainable; further unscientific exploitation of natural resources is increasing the environmental degradation in the region (Singh, 2006). Proper policies and their implementation for harnessing the potential of the natural resources is the need of the hour for the sustainable development in the region.

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Natural Polyphenols and its Potential in Preventing Diseases Related to Oxidative Stress as an Alternative Green Nutraceutical Approach

Sreenivasan Sasidharan^{1,*}, Shanmugapriy¹, Subramanion Lachumy Jothy¹, Mei Li Ng², Nowroji Kavitha¹, Chew Ai Lan¹, Khoo Boon Yin¹, Soundararajan Vijayarathna¹, Leow Chiuann Herng¹ and Chern Ein Oon¹

¹Institute for Research in Molecular Medicine, Universiti Sains Malaysia, 11800 USM, Penang, Malaysia; ²Integrative Medicine Cluster, Advanced Medical and Dental Institute (AMDI), Universiti Sains Malaysia, 13200, Kepala Batas Penang, Malaysia;

*Correspondence: srisasidharan@yahoo.com; Tel: +60 46534820

Abstract: Green bioactive polyphenol from Mother Nature especially from medicinal plants are a rich source of novel therapeutics. Therefore, the search for bioactive molecules from nature continues to play an important role in the invention of new medicinal agents. Most plants do provide an array of phytochemicals that may contribute to reduction of disease and slowing of aging. Oxidative stress which is continuously produced *in vivo* by oxygen-centred free radicals and other reactive oxygen species may leads to various diseases. Since the oxidative stress has a great impact on the human health, it is appropriate to examine the role of natural antioxidant as a defence system. In this line, medicinal plant based natural antioxidants such as polyphenols with free radical scavenging activity are emerging as the primary components of holistic approaches in impeding adverse effect of oxidative stress. This chapter focuses on biological effects of natural polyphenols on oxidative stress and related diseases as an aalternative green nutraceutical approach.

Keywords: Free radicals; medicinal plant; natural antioxidants; oxidative stress; polyphenol

1. Introduction

Oxidative damage caused by free radicals to macromolecules outlines the foundation of what is arguably the most popular current explanation of ageing related diseases (Lawrence *et al.*, 2005). Recent years have seen a surge of interest in the role of mitochondrial dysfunction, reactive oxygen species production and mitochondrial DNA mutation as driving factors in the ageing and various diseases (Balaban *et al.*, 2005; Trifunovic *et al.*, 2005; Bender *et al.*, 2006; Passos *et al.*, 2007). Hypothesized links between aging and oxidative stress are not new and were proposed over 50 years ago (Harman, 1956). Mitochondria are found in nearly

all eukaryotes. They vary in number and location according to cell type. Conversely, numerous mitochondria are found in human liver cells, with about 1000–2000 mitochondria per cell, making up 1/5 of the cell volume (Alberts *et al.*, 1994). Given the role of mitochondria as the cell's powerhouse, there may be some leakage of the high-energy electrons in the respiratory chain to form reactive oxygen species. This was thought to result in significant oxidative stress in the mitochondria with high mutation rates of mitochondrial DNA (mtDNA) (Richter *et al.*, 1988). A vicious cycle was thought to occur, as oxidative stress leads to mitochondrial DNA mutations, which can lead to enzymatic abnormalities and further

oxidative stress. The accumulation of these damaged macromolecules is proposed to contribute significantly to aging and various diseases (Ames *et al.*, 1993). Hence, plants polyphenol with free radical scavenging activities are likely to make a considerable contribution for the protection against the free radical oxidation. Bioactive products from Mother Nature are a rich source of novel therapeutics. Therefore, the search for bioactive molecules from nature continues to play an important role in the invention of new medicinal agents. Most plants do provide an array of phytochemicals that may contribute to reduction of diseases and slowing of aging. This chapter focuses on biological effects of natural polyphenols on oxidative stress and related diseases as an alternative green nutraceutical approach.

2. What are free radicals?

The human body is, composed of different types of living cells which are organized into tissues, organs, and systems. Cells are made of different types of molecules which consist of atoms. Atoms comprise of a nucleus, neutrons, protons and electrons. Protons are positively charged particles in the nucleus that determine the number of negatively charged particles known as electrons in the atomic orbital.

An atom's chemical behaviour is largely dictated by the number of electrons in its outermost shell. An atom is stable when its outermost shell is full. A free radical is an atom or group of atoms that has an unpaired electron due to splitting of weak bonds between electrons on the outermost shell and is therefore unstable and highly reactive. It will attempt to stabilize itself by reacting with another atom or molecule to donate its electron or to aggressively capture an electron to fill its outermost shell, thus stimulating a cascade of free radicals when the attacked atom or molecule receives an extra electron or loses its electron (Halliwell, 1993).

Reactive nitrogen species (RNS) and reactive oxygen species (ROS) are free radicals that arise from normal cellular metabolism or as a consequence to pathological exposures. Nitric oxide, peroxynitrite and nitrogen dioxide are nitrogen-containing oxidants, often referred to as reactive nitrogen species. ROS are *reactive* molecules derived from oxygen molecules such as superoxide, hydroxyl, peroxy, alkoxy and singlet oxygen. They are usually generated as by-products in the mitochondria, peroxisomes, cytochrome P450 and other organelles. Free radicals include reactive oxygen and nitrogen species which are also collectively termed as reactive oxygen nitrogen species (RONS). RONS are known for being both beneficial and harmful. On the good side, they have been reported to have a crucial role in cell signalling (Adams *et al.*, 2015; Reczek and Chandel, 2014), homeostasis (Kuster *et al.*, 2010; Shadel and Horvath, 2015) and immune defence in response to inflammatory stimuli (Mizgerd and Brain, 1995; Reshi *et al.*, 2014).

However, these radicals are over-generated in response to unfavourable environmental conditions such as poor nutrition, stress, smoking, alcohol, exercise, radiation, inflammation, drugs or exposure to air pollutants and chemicals (Figure 1). At high concentrations, free radicals have the ability to alter proteins, carbohydrates, lipids and nucleic acids thus impairing cellular functions and leading to pathogenesis of cancer, aging, diabetes, atherosclerosis and other inflammatory diseases (Pham-Huy *et al.*, 2008).

Antioxidants are electron donors that can neutralize free radicals, thus preventing them from causing cellular damage (Figure 1). A balance between free radicals generated and antioxidant protective defence system is required for optimal physiological function (Bouayed and Bohn, 2010). Oxidative stress or nitrosative stress occurs when there is an imbalance between the production of free radicals and the ability of the body neutralize the deleterious effects through the role of

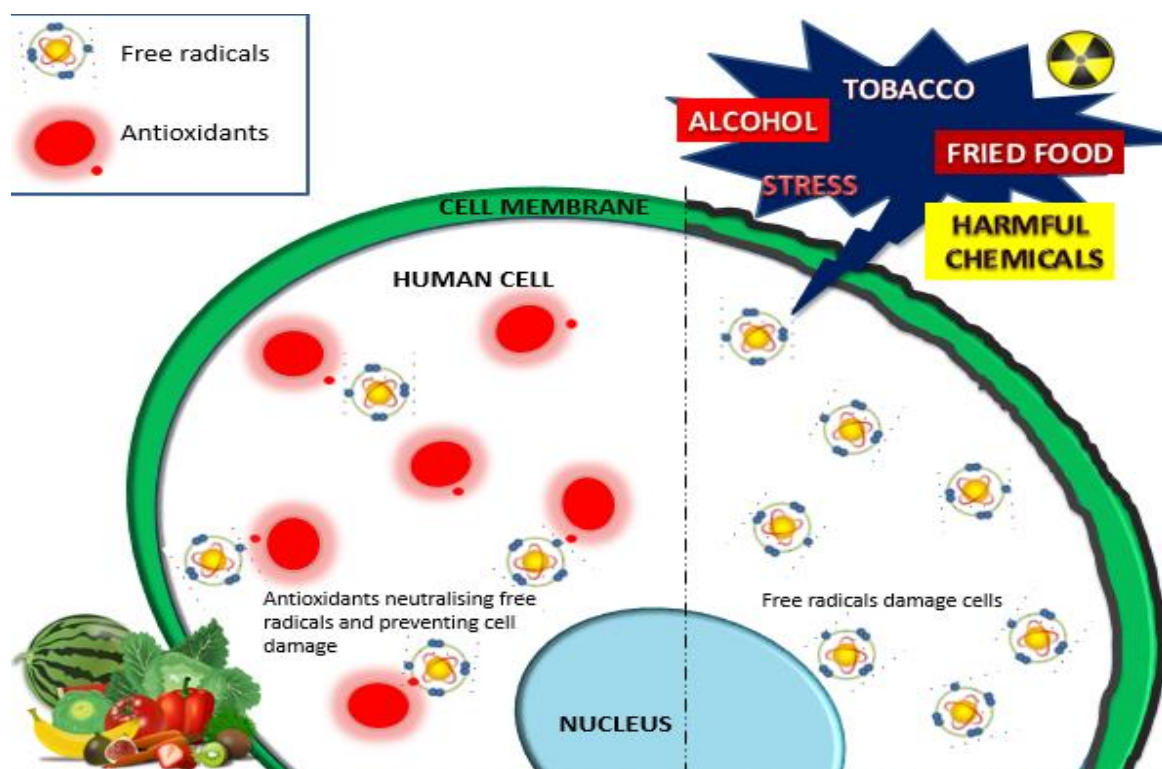


Figure 1: Free radicals are generated due to factors such as stress, alcohol, tobacco, harmful chemicals including pesticides and drugs, fried food and radiation. Found abundantly in natural food, antioxidants are free radical scavengers that react with free radicals to neutralize them. Antioxidants function by donating an electron to the free radical before the latter oxidizes other components within the cell. The free radical is stabilized and becomes non-damaging to cells once it receives a free electron from the antioxidant.

antioxidants. The human body manufactures endogenous antioxidant enzymes in order to control these destructive free radical chain reactions (Valko *et al.*, 2007). However, the body also relies on dietary antioxidants to fulfil the needs of other antioxidants it cannot produce (Diplock *et al.*, 1998), such as those found abundantly in bioactive food components including fruits, vegetables, grains and mushrooms. Examples of dietary antioxidants include lycopene, beta-carotene, lutein, zeaxanthin, alpha-tocopherol, vitamin A and vitamin C.

3. Oxidative stress

The origin of term 'oxidative stress' from its very nature defined as the combination of electron transfer, free radicals, oxygen metabolites such as the superoxide anion radical, hydrogen peroxide, hydroxyl radical and singlet molecular oxygen with a biological concept of stress

was first introduced by Selye (1955). A continuously activity of human body reacts with oxygen when breathing resulted in energy production by cells and generate highly reactive molecules within the cells known as free radicals. The effect exerted by free radicals in the body is called 'oxidative stress' (Finaud *et al.*, 2006).

Since overwhelming research has been developed, the contemporary concept of 'oxidative stress' was updated and briefly redefined as disturbance in the balance between oxidants production and antioxidants defences (Sies and Jones, 2007; Sies, 2015) which are associated with electron transfer influencing the redox state of cells and organism. Subsequently, the imbalance redox mechanism leads to the production of reactive oxygen species (ROS) that includes free radicals (superoxide, hydroxyl radical, peroxy, alkoxyl, and hydroperoxyl) and non-free radicals (hydrogen peroxide, hypo-

chlorous acid, ozone, singlet oxygen) are mainly involved in growth, differentiation, progression and death of the cells. (Rahman *et al.*, 2012; Rajendran *et al.*, 2014).

Free radicals known as small molecules and unpaired electron amid highly reactive proliferation characteristic which differ from most biological molecules that tend to involve in initiate chain reactions from single free radical until propagate to damage multiple molecules. Oxidative stress occurs when excessive free radical formation within a cell or organism as a form of ROS due to aerobic metabolism and immune activation (Delmastro-Greenwood and Piganelli, 2013), UV radiation (Halliwell and Gutteridge, 2015), heme-oxygenase accumulation (Vanella *et al.*, 2013), and hypoxia (Yang *et al.*, 2011). Failure of the cell's defence mechanisms to neutralize or balance the accumulation of free radicals may leads to mitochondrial dysfunction, DNA damage and lipid peroxidation can trigger programmed cell death pathways (Dixon and Stockwell, 2014). The findings could explain that low concentration of ROS productions play role in intracellular signalling and defence against pathogens, while the higher concentration of ROS has been linked to clinically relevant diseases including cancer, cardiovascular disease, asthma, ischemia, diabetes and neurodegenerative diseases (Reynolds *et al.*, 2007; Halliwell and Gutteridge, 2015; Phaniendra *et al.*, 2015). Mitochondria is a major part of cellular sources of ROS which consume oxygen with the process of oxidative phosphorylation. Excessive formation of free radicals is known to weaken defence mechanisms against oxidation and it results in more cell damages (Zorov, 2014). Consequently, the increasing level of oxidative stress involved in pathological redox reaction, process of ageing which can initiate tissue damage via apoptosis and necrosis (Cui *et al.*, 2012).

4. Oxidative stress and immune response

Immune system is important for our body to protect from the invasive pathogens. The human defence system has been well developed and can be divided into two immunity reactions, namely innate immunity and adaptive immunity. As its name suggests, the innate immunity is a non-specific first barrier of defence which is able to act fast towards microbial invasion. The adaptive immunity, on the other hand, is a highly specific barrier of defence due to possess immunological memory function that can rapidly and efficiently remove the pathogens that invaded into body (Poland *et al.*, 2013). Components of the adaptive immune system are normally silent; however, when activated, these components 'adapt' to the presence of infectious agents by activating, proliferating, and creating potent mechanisms for neutralizing or eliminating the microbes. In general, there are two types of adaptive immune responses where humoral immunity, mediated by antibodies produced by B lymphocytes (B-cells), and cell-mediated immunity, mediated by T lymphocytes (T-cells). Unlike B-cells, T-cells recognize circulating antigens of many chemical structures, the vast majority of T cells (> 95%) are only able to recognize peptide fragments that are displayed by specialized molecules, MHC molecules, on the surfaces of antigen presenting cells. Therefore, this system ensures that T-cells are able to recognize antigens that might be floating in the cytosol or contained within ingested vesicles of various cells (Thomas *et al.*, 2013).

An imbalance between reactive oxygen species (ROS) and reducing agents (antioxidants) towards a pro-oxidant state can always result to oxidative stress (Sies, 1997). The damaging of macromolecular in the form of protein carbonylation, lipid peroxidation and DNA oxidation has historically has been proven as harmful to particular functional

cells and being part of the main factors to hypertension and aging problems (Annor *et al.*, 2015; Zhao *et al.*, 2017). Up to date, ROS is widely recognised as signalling molecular substances (Suzuki and Sevanian, 1997) that enable to trigger a wide range of biological effects. With regard to the immune system, high levels of ROS can also be beneficial. For instances, neutrophils generate ROS and release them intracellularly and extracellularly in the form of an “oxidative burst” to defend against and destroy pathogens, thus providing antimicrobial protection (Dahlgren and Karlsson, 1999). However, excessive ROS are generated in the presence of immune complexes with auto-antigens where further macromolecular damage is induced. Prolonged exposure to high ROS concentrations can inhibit T-cell proliferation and lead to apoptosis (Thoren *et al.*, 2007), and incubation of T-cells with the reactive nitrogen species (RNS) peroxynitrite can also inhibit their proliferation (Kasic *et al.*, 2011). Previous study has revealed that different T-cell responses to ROS production may be due to the extent of change to the cellular redox environment (Griffiths, 2005). In some cases such as absence of antigen presenting cell, reactive carbonyls including 4-hydroxy-2-nonenal and malondialdehyde (MDA), which are generated on proteins and lipids randomly in the presence of ROS, will promote differentiation towards a Th2 phenotype (Moghaddam *et al.*, 2011). These data emphasize that the importance of ROS homeostasis and flux in governing cell maturation and that the balance between oxidising and reducing agents is a delicate process which must be tightly regulated and well managed, depending on whether the requirement is for protecting against bacteria, in an immune response, or requirements for T-cell signalling, activation and regulation of function. Owing to their pivotal role, the effect of the oxidative stress on T-cell biochemistry and its implications in autoimmune disease such as rheumatoid arthritis (RA)

are greatly debated in healthcare management.

5. Oxidative stress and cancer

Oxidative stress is closely related to all aspects of cancer, from carcinogenesis to the tumor-bearing state, from treatment to prevention. Cancer cells generally display elevated ROS level compared to normal cells that give them a proliferative advantage and promote malignant progression. The excess level of ROS typically observed in cancer cells are the result of accumulation of intrinsic and environmental factors. In cancer cells, high levels of ROS can result from hypoxia, mitochondrial dysfunction, peroxisome activity, enhanced cellular metabolic activity, increased cellular receptor signaling, oncogene activity, increased activity of oxidases, cyclooxygenases, lipoxigenases and thymidine phosphorylase, and the crosstalk between cancer cells and immune cells recruited to the tumor site (Holmström and Finkel, 2014). Environmental sources of ROS that can significantly contribute to tumorigenesis include ionizing radiation, xenobiotics, tobacco components, chlorinated compounds, barbiturates, metal ions and phorbol esters etc. Figure 2 illustrates the potential outcomes when such ROS level exceeds the capacity of the oxidation-reduction system of the cell, may cause DNA, protein and lipid damage, leading to cellular responses such as chromosomal instability, genetic mutation, alterations in cellular metabolism and modulation of cell growth that may lead to malignant transformation by affecting crucial hallmarks of cancer.

When present at high and sustained levels, ROS can cause severe deleterious modifications to DNA, protein, and lipids. During the initiation stage, ROS may produce DNA damage by introducing gene mutations and structural alterations into the DNA. ROS-induced DNA damage can result in single- or double-strand breakage, base modifications,

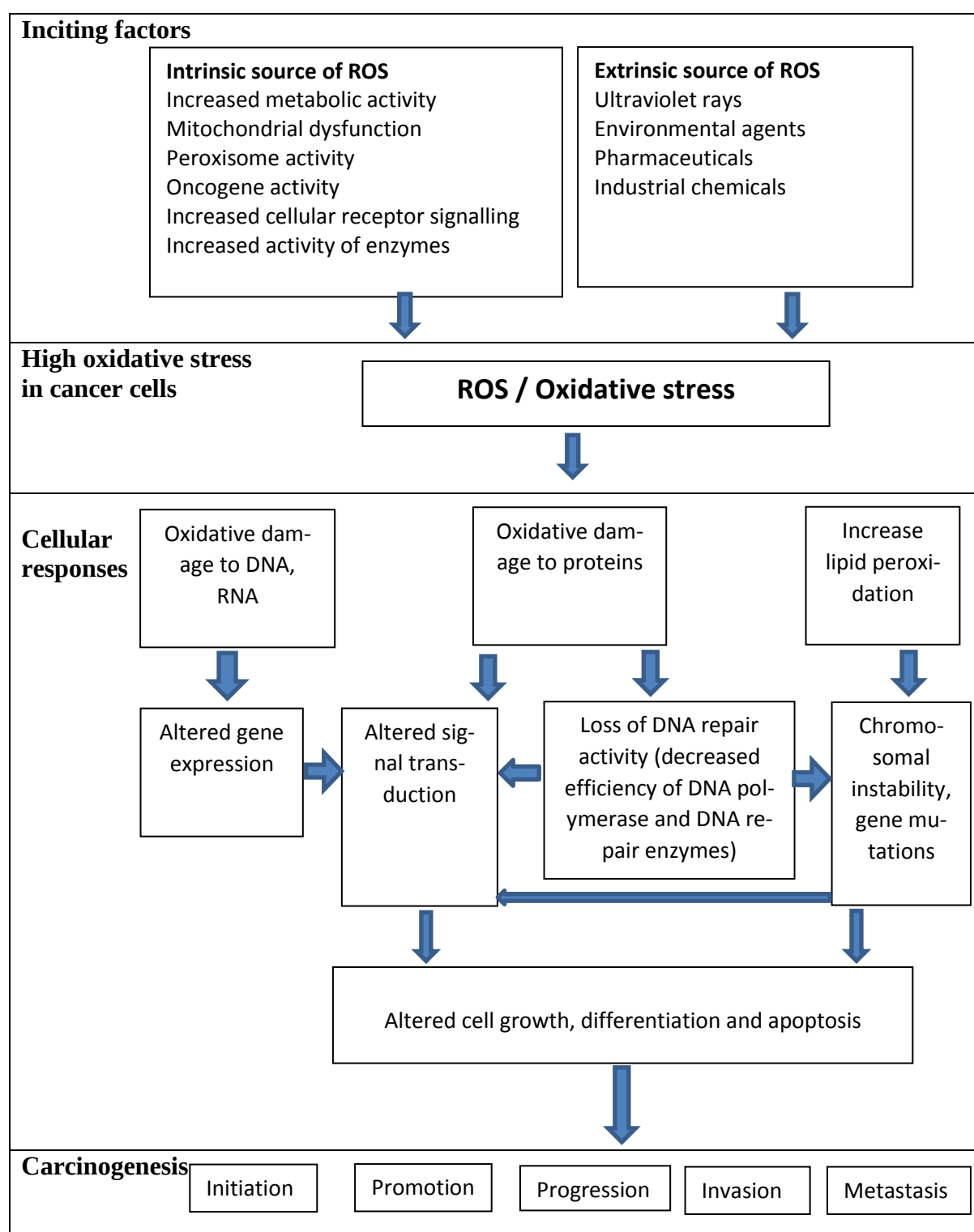


Figure 2: ROS and their role in the development of human cancer.

deoxyribose modification and DNA cross-linking. Cell death, DNA mutation, replication errors and genomic instability can occur if the oxidative DNA damage is not repaired prior to DNA replication (Valko *et al.* 2006). Oxidative DNA damage/repair imbalance due to loss of DNA repair activity can lead to disease-causing

mutations and may contribute to increased risk of carcinoma (Caramori *et al.*, 2011). Proteins and lipids are also significant targets for oxidative attack and modification of these molecules can increase the risk of mutagenesis. Lipid peroxidation results in production of reactive metabolites which demonstrate high reactivity

with protein and DNA and have been implicated in the pathogenesis of cancer (Tuma, 2002). ROS also contribute to chromosomal instability and mutation through its role in increasing the rates of mutation, enhancing sensitivity to mutagenic agents and compromising the surveillance systems. In addition to inducing DNA, lipid and protein damage, oxidative damage to protein-coding or –non coding RNA may potentially cause errors in protein synthesis or dysregulation of gene expression. ROS-induced alteration of gene expression can occur through modulation of a host of signaling pathways including cAMP-mediated cascades, calcium-calmodulin pathways and intracellular signal transducers such as nitric oxide (Bertin and Averbeck, 2006). ROS stimulation on signal transduction pathways can lead to activation of key transcription factors such as Nrf2 and NF- κ B.

ROS interact with the multistage processes in carcinogenesis including initiation, promotion, proliferation, invasion, angiogenesis and metastasis. ROS act as essential signalling molecules and modulate a number of redox-sensitive signalling pathways in initiation of carcinogenesis. Well-characterized targets include ROS-mediated regulation of the mitogen-activated protein (MAP) kinase/Erk cascade, phosphoinositide-3-kinase (PI3K)/Akt-regulated signaling cascades, as well as the I κ B kinase (IKK)/nuclear factor κ -B (NF- κ B)-activating pathways (Ray *et al.*, 2012). Oxidative stress-mediated signaling pathways are persistently elevated in many types of cancers and affect all characters of cancer cell behavior, where they participate in cell growth/proliferation, differentiation, protein synthesis, glucose metabolism, cell survival and inflammation (Storz, 2005).

In promotion stage, ROS can contribute to abnormal gene expression, inhibition of intercellular communication and modification of second-messenger systems, thus resulting in an increase in cell proliferation or a decrease in apoptosis of the initiated cell population (Klaunig and

Kamendulis, 2007). Most initiating mutations affect proto-oncogenes or tumor suppressor genes. Proto-oncogenes code for a variety of growth factors, growth factor receptors, enzymes or transcription factors that promote cell growth and cell division while oncogenes are mutated versions of proto-oncogenes that promote abnormal cell proliferation. Activation of oncogenes and loss of tumor suppressors cause alterations to multiple intracellular signaling pathways that promote metabolic reprogramming in cancer, resulting in enhanced nutrient uptake to supply energetic and biosynthetic pathways for enhanced growth and survival (Zhang *et al.*, 2013).

Oxidative stress may participate in the progression stage of the cancer process by adding further DNA alterations to the initiated cell population (Qanungo *et al.*, 2005). It can also promote many aspects of tumor development and progression through various biological processes. It acts on cellular proliferation through extracellular-regulated kinase 1/2 (ERK1/2) activation and ligand independent receptor tyrosine kinases (RTK) activation. ROS were shown as positive regulators of tumor cell proliferation by modulating key proteins in cell cycle progression such as cyclin, ATM (ataxia telangiectasia mutated) and antioxidant enzymes like MnSOD, catalase and glutathione peroxidase (Browne *et al.*, 2004; Lewis *et al.*, 2005). ROS are involved in Anoikis resistance, PTEN inactivation, activation of Src, NF- κ B, CREB and phosphatidylinositol-3 kinase (PI3K)/Akt, enabling the tumor cells to escape from apoptosis (Giannoni *et al.*, 2009; Zhu *et al.*, 2011). In invasion and metastasis, ROS play a role in Met over-expression, matrix metalloproteinase secretion into the extracellular matrix (ECM), invadopodia formation, Rho-Rac interaction, plasticity in cell motility and epithelial–mesenchymal transition (EMT). These help cancer cells to escape the primary tumor, invade matrix of different organs, find a suitable metastatic niche and then

grow in the secondary site. In addition, oxidative stress is involved in continuous angiogenesis through its role in endothelial progenitor activation, release of VEGF and angiopoietin and recruitment of perivascular cells.

6. oxidative stress (os) in diabetes pathology

A growing body of evidence suggest that increased oxidative stress and deficit in antioxidant defense mechanism are central players in pathogenesis of diabetes complications, in particular β -cell dysfunction and failure (Folli *et al.*, 2011). Under physiological condition, reactive oxygen species (ROS) serve as second messenger regulates signal transduction and gene expression. Oxidative stress develops from imbalance in redox homeostasis (overproduction of mitochondrial reactive oxygen species (ROS) that exceeds the level of antioxidants) leads to aberrant β -cell function and apoptosis. ROS are heterogenous molecules comprises of free radicals, such as nitric oxide ($\text{NO}^\bullet$), superoxide ($\text{O}_2^{\bullet-}$), hydroxyl radical ($\text{OH}^\bullet$), non-radicals such as hydrogen peroxide (H_2O_2), anions such as superoxide (O_2^-) and peroxyxynitrite (ONOOK) (Chang *et al.*, 1993; Pieper *et al.*, 1997; Lenzen, 2008; Newsholme *et al.*, 2012; Cao and Kaufman, 2014; Keane *et al.*, 2015). Sources of free radicals production include the mitochondrial electron transport system, NADPH oxidases, xanthine oxidase (primary source in cardiomyocytes), uncoupled nitric oxide synthase (NOS) and arachidonic acid (primary source in vascular cells) pathway. Mitochondria are major source of free radicals production in cells. ROS was noted as a key upstream signaling event mediates downstream metabolic pathways, leading to loss of cellular biological function and ultimately cell death (Brownlee, 2005). Ample evidence indicate that ROS damage plays a major role in pathogenesis of micro- (diabetic retinopathy, nephropathy, and neuropathy) and cardiovascular complica-

tions (atherosclerotic diseases affecting arteries that supply heart, brain and lower extremities) (Greene *et al.*, 1992; Rosen *et al.*, 2001) and onset of diabetes (Kayama *et al.*, 2015). Vincent and colleagues has demonstrated that ROS production and neuron injury are activated within 1-2 hours of hyperglycaemic insult. Majority of the patients with impaired glucose tolerance have significant peripheral neuropathy, suggesting that ROS induced by hyperglycaemia is critical to cause major diabetes complications (Vincent *et al.*, 2002).

Metabolic abnormalities such as hyperglycaemia, hyperlipidaemia, increased free fatty acids, insulin resistance and hyperinsulinaemia, each of which was noted to induce oxidative stress in endothelial cells of the blood vessels and myocardium. In addition, genetic susceptibility of an individual and presence of accelerating factors (e. g. hypertension and dyslipidaemia) also contribute to development of diabetes complications (general features of chronic hyperglycaemia-induced tissue damage are depicted in Figure 3). Several large scale perspective studies, such as the (Diabetes Control and Complication Trial DCCT/EDIC (The Diabetes Control and Complications Trial Research Group, 1993), UK prospective Diabetes Study (UKPDS) (UK Prospective Diabetes Study (UKPDS) Group, 1998), and Steno 2 Study have concluded that chronic hyperglycaemia as a key risk factor underlying diabetes pathology (Gaede *et al.*, 2008). Hyperglycaemia is known to trigger oxidative stress through FIVE major molecular mechanisms (Figure 4 depicts the mechanism underlying oxidative stress and diabetes pathology): (1) Activation of Polyol pathway (2) Increased intracellular advanced glycation end products (AGEs) pathway activity and receptor expression for AGEs (RAGE) (3) Activation of Protein Kinase C isoforms (PKC) (4) Increased Hexosamine pathway flux (5) Decreased antioxidant defenses (Sima *et al.*, 1990; Engerman *et al.*, 1994; Brown-

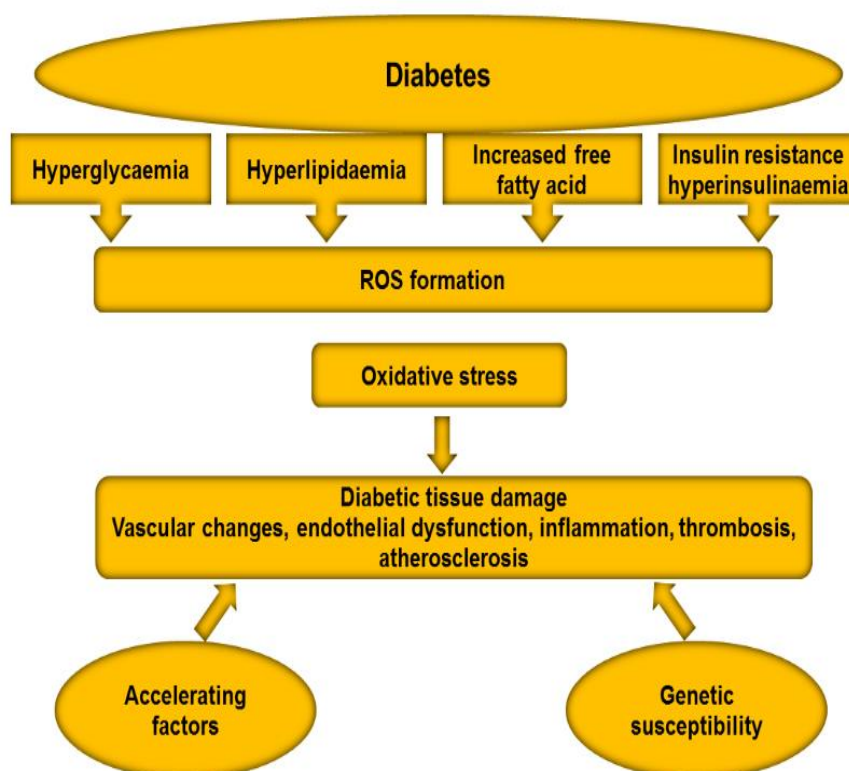


Figure 3: General features of chronic hyperglycaemia-induced diabetic tissue damage (Giacco and Brownlee, 2010).

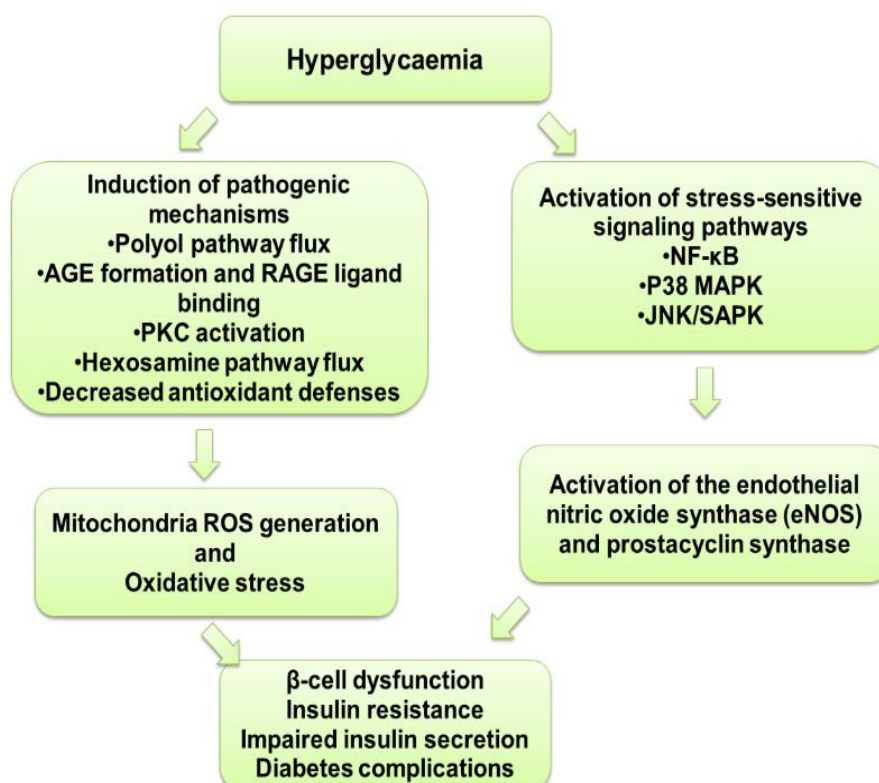


Figure 4: Mechanisms underlying hyperglycaemia-induced pathophysiology of diabetes via the generation of ROS and activation of stress-sensitive signaling pathways. Each mechanism is discussed in the text (Vincent *et al.*, 2004).

lee, 1995; Lee *et al.*, 1995; Ganz and Seftel, 2000; Brownlee, 2005). The effect of oxidative stress damage is aggravated by inactivation of anti-atherosclerotic enzymes (endothelial nitric oxide synthase (eNOS) and prostacyclin synthase. In addition, oxidative stress also activates stress-sensitive signaling pathways, such as nuclear redox sensitive transcription factor (NF- κ B), p38 MAPK, and NH2-terminal Jun kinases/stress-activated protein kinases (JNK/SAPK) leads to both insulin resistance and impaired insulin secretion (Folli *et al.*, 2011; Brownlee, 2001).

7. Molecular mechanisms of hyperglycaemia-induced oxidative stress in diabetes

Hyperglycaemia-induced activation of polyol pathway was the first mechanism discovered (Gabbay *et al.*, 1966). This pathway has been therapeutic target for diabetes neuropathy (Oates and Mylari, 1999). Recent human genetic study has implicated polymorphisms of the aldose reductase gene associated with increased risk for diabetes complications (Oates and Mylari, 1999). Excess glucose activates polyol pathway. Aldose reductase (dependent upon NADPH as co-factor) increases conversion of glucose to polyalcohol sorbitol. Excessive activation of polyol pathway results in depletion of intracellular NADPH and GSH which is an important intracellular antioxidant (Lee and Chung, 1999). Accumulation of sorbitol forms cellular osmotic stress (Stevens *et al.*, 1993).

Excess glucose induces auto-oxidation through activation of the AGEs pathway.

The AGE precursor damages cells by three mechanisms: modification of proteins involve in gene transcription (Giardino *et al.*, 1994; Shinohara *et al.*, 1998), modification of extracellular matrix molecules (McLellan *et al.*, 1994), and modification of circulating protein in the blood (e. g. albumin) (Vlassara *et al.*,

1988; Li *et al.*, 1996). These results in auto-oxidation of glucose to glyoxals, decomposition of the Amadori product (glucose-derived 1-amino 1-deoxyfructose lysine adducts, to 3-deoxyglucosone, and fragmentation of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate to methylglyoxal (Brownlee, 2001). In addition, increased AGEs production promotes the binding of AGEs to its receptors (RAGE). Binding of AGEs to RAGE induces overproduction of ROS and activation of NF- κ B signaling and upregulation of intracellular adhesion molecule-1(ICAM-1), vascular adhesion cell molecule-1 (VCAM-1), monocyte chemotactic protein-1 (MCP-1), PAI-1, tissue factor, and VEGF (Yamagishi *et al.*, 1997; Bierhaus *et al.*, 2001).

Previous studies demonstrated that PCK activity was increased in the retina, kidney and microvasculature of diabetic rats (Craven, P.A. and F.R. DeRubertis, 1989; Lee *et al.*, 1989), suggested that the lipolytic pathway and production of diacylglycerol induces PKC activation (Ishii *et al.*, 1998). Hyperglycaemia increases diacylglycerol synthesis, which is a critical activating co-factor for PKC isoforms (Derubertis and Craven, 1994; Xia *et al.*, 1994; Koya *et al.*, 1997; Koya and King, 1998). PKC activation has been shown to have diverse effects on gene expression in different cell types. PKC activation inhibits insulin-stimulated endothelial Nitric Oxide Synthase (eNOS) expression in the endothelial cells and decreases nitric oxide production in the smooth muscle cells (Vlassara *et al.*, 1995). In vascular smooth muscle cells, PKC activation induces over-expression of fibrinolytic inhibitor, plasminogen activator inhibitor (PAI-1) and activation of NF- κ B (Abordo and Thornalley, 1997). PKC enhances accumulation of microvascular matrix protein by up-regulation of transforming growth factor (TGF- β), fibronectin and type 4 collagen in both culture mesangial cells and glomeruli of diabetic rats (Doi *et al.*, 1992). PKC also enhances vascular permeability by increas-

ing the expression of vascular endothelial growth factor (VEGF) (Skolnik *et al.*, 1991).

Lastly, hyperglycaemia causes damage to the blood vessel through activation of hexosamine pathway. The end product of this pathway, uridine diphosphate (UDP)-N-acetyl glucosamine regulates gene expression implicated in vascular complications (such as PAI -, TGF- α , TGF- β 1). In addition, activation of hexosamine pathway impairs Insulin Receptor Substrate (IRS)/phosphatidylinositol 3-kinase (PI3-K)/Akt pathway, resulting in deregulation of eNOS activity (Bucala *et al.*, 1991; Kolm-Litty *et al.*, 1998).

8. Oxidative stress and β -cell dysfunction in diabetes

Diabetes mellitus (DM) is characterized by failure of the pancreatic β -cells to maintain glucose homeostasis. Physiologically, the pancreatic β -cells secrete hormone insulin and regulate glucose homeostasis. Insulin drives glucose uptake in the liver (reducing hepatic gluconeogenesis both directly and in conjunction with suppression of glucagon secretion), muscle and fat (Könnner, 2007). Because of their high biosynthetic load and requirement for oxygen, pancreatic β -cells are very vulnerable to oxidative stress (Lenzen, 2008; Newsholme *et al.*, 2012; Cao and Kaufman, 2014; Kaneto and Matsuoka, 2015).

Oxidative stress and endoplasmic reticulum stress (ER) are key pathological features in particular type 2 diabetes mellitus (T2DM), contribute to pancreatic β -cell dysfunction, inducing inflammation (immune activation) and β -cell apoptosis. Previous studies have suggested the oxidative stress is able to suppress insulin transcription and associated with accumulation of β -amyloid in the human pancreatic islet (Kaneto and Matsuoka, 2015). In obesity and early stage of diabetes, nutrient overload leads to development of mild insulin resistance and hyperglycaemia. Increased insulin production by the pan-

creatic β -cells triggers oxidative stress and ER stress, exacerbated by high circulating glucose and lipids (non-esterified fatty acid). Oxidative stress and ER stress induce chemokine production and activates inflammatory cells in the pancreatic islet. In turn, the activated inflammatory cells produce cytokines that further exacerbate oxidative and ER stress and disrupt β -cell secretory pathway function. In addition, oxidative stress induces unfolded protein response (UPR) and NF- κ B activation. In early diabetes (manifested by chronic ER stress and inflammation), increased proinsulin:insulin ratio impairs insulin signaling further aggravates hyperglycaemia. Overall, this vicious cycle leads to β -cell apoptosis and progression to diabetes (summarized schematically in Figure 5). Several mechanisms have been implicated in β -cell apoptosis (Nakagawa *et al.*, 2000; Oyadomari *et al.*, 2002; Puthalakath *et al.*, 2007; Song *et al.*, 2008; Mahdi *et al.*, 2012; Supale *et al.*, 2012; Uruno *et al.*, 2015). The PERK/ATF4-mediated activation of CHOP and IRE1a/TRAF2/ASK1-mediated activation of JNK are important molecular mechanisms (reviewed in Papa FR 2012) (Papa, 2012). A growing body of evidence suggests that ER stress induces autophagy (an important mechanism for removal of terminally misfolded protein from the endoplasmic reticulum (ER) leads to induction of apoptosis (Wang *et al.*, 2014; Li *et al.*, 2012; Quan *et al.*, 2012). Another mechanism involves NF- κ B and interleukin 1 beta (IL1b) activation (reviewed in Hasnain SZ *et al.*, 2012; Hasnain *et al.*, 2014).

9. Current therapeutics in diabetes

Good glycaemic control is the most effective mean of mitigating diabetes complications in particular type 1 diabetes (Greene *et al.*, 1992; Molitch *et al.*, 1993). In general, drug available for diabetes work by reducing stress on β -cells biosynthesis pathway. In diabetes patients, the use of drug that promotes insul-

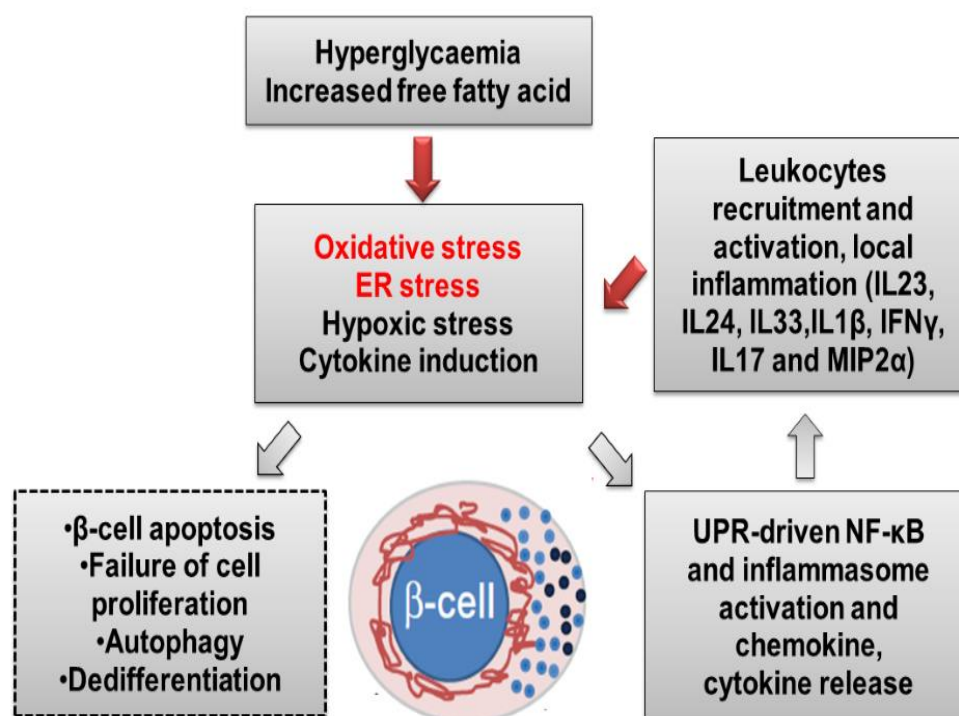


Figure 5: Schematic representation of the cycle of oxidative and ER stress and its effects on glucose homeostasis in diabetes (Hasnain *et al.*, 2015).

-in secretion (such as sulfonylureas) is known to causes loss of β -cell function. Another class of GLP-1 receptor agonist, which promotes insulin secretion in a glucose dependent-manner, may also have long-term damaging effect on β -cell (Hasnain *et al.*, 2014). Drug that suppresses gluconeogenesis (metformin), increase glucose excretion (SGLT-2 inhibitor) or reduces peripheral insulin resistance (thiazolidinediones) or exogenous insulin.

Given that pronounced oxidative stress mediates major diabetes complications, antioxidant therapy remains a novel therapeutic approach for diabetes patients. Antioxidant drugs target NADPH oxidases are unable to combat high level of oxidative stress (Li *et al.*, 2012). In view that the IL22 receptor is the most highly expressed in human pancreatic islet cells, studies have been identifying IL22 as novel antioxidant target in diabetes (Cobleigh and Robek, 2013; Kumar *et al.*, 2013; Rutz *et al.*, 2013; Hasnain *et al.*, 2014; Sabat *et al.*, 2014).

10. Biological effects of natural polyphenols on oxidative stress

Oxygen is an essential element of life used by cells to generate energy in the form of ATP whereby this process occurs within the mitochondria (Turrens, 2003). The process, however, causes the production of free radicals, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) due to the cellular redox process in the cells (Pham-Huy *et al.*, 2008). At low or moderate concentration, these species exert beneficial effects on cellular responses and immune function, but when the species exist at higher levels, oxidative stress is generated (Young and Woodside, 2001; Halliwell, 2007; Pham-Huy *et al.*, 2008). Oxidative stress refers to the balance between the production of free radicals and antioxidant defences in a cell (Betteridge, 2000). The mechanism arises when there is an unfavourable balance between the free radical production and antioxidant defences, which result in the damage of a broad range of molecular species, including li-

pids, proteins and nucleic acid in the cells (Rock *et al.*, 1996; McCord, 2000).

The concept of oxidative stress was first hypothesised in the 1950s by researchers that investigated the toxic effects of ionizing radiation, free radicals, and the similar toxic effects of molecular oxygen (Gerschman *et al.*, 1954), as well as its possible contribution to the aging process (Harman, 1956). Interest in this field of research grew (Hybertson *et al.*, 2011) when studies reported that the biological systems are capable of producing substantial amounts of superoxide free radical, O_2^- through the natural metabolic pathways (McCord & Fridovich, 1968) and the activity of antioxidant enzymes, superoxide dismutases (SODs) is necessary for the survival of aerobic organisms (McCord and Fridovich, 1969; McCord *et al.*, 1971). Besides the aging process, oxidative stress has been postulated to play a role in many other conditions, as well. These conditions includes inflammatory diseases (arthritis, vasculitis, glomerulonephritis, lupus erythematosus, adult respiratory diseases syndrome), ischemic diseases (heart diseases, stroke, intestinal ischemia), hemochromatosis, acquired immunodeficiency syndrome, emphysema, carcinogenesis, gastric ulcers, hypertension and preeclampsia, neurological disorders (Alzheimer's disease, Parkinson's disease, muscular dystrophy), alcoholism and smoking-related diseases (Pham-Huy *et al.*, 2008; Lobo *et al.*, 2010).

To protect cellular components from the free radical-induced damage, the body possess several mechanisms to counteract oxidative stress by producing an extensive range of antioxidant, both endogenous (generated in situ) and exogenous (supplied through food, e.g. polyphenols) in the cells. The antioxidants can be divided into three main categories which are antioxidant enzymes, chain breaking antioxidants and transition metal binding proteins (Young and Woodside, 2001; Pham-Huy *et al.*, 2008).

Polyphenols are a large group of natural antioxidants found mostly in fruits, vegetables, cereals and beverages (Arts and Hollman, 2005; Pandey and Rizvi, 2009). There are more than 8,000 polyphenolic compounds that have been identified in various plant species, which arise from a common intermediate, phenylalanine or an immediate precursor, and shikimic acid. Polyphenols contain phenol rings in the basic structure. Based on the number of phenol rings and the basis of the structural elements that binds to these rings, polyphenols can be classified as phenolic acids, flavonoids, stilbenes and lignans (Spencer *et al.*, 2008; Pandey and Rizvi, 2009). Epidemiological studies have shown that high consumption of polyphenols lowered the risk of chronic human diseases (Scalbert *et al.*, 2005; Arts and Hollman, 2005). Besides that, the consumption of polyphenols have also been linked with cardio-protective effect, anti-cancer effect, anti-diabetic effect, anti-aging effect and neuroprotective effect (Pandey and Rizvi, 2009). Several types of research have also concluded that high phenolic content correlates with a good antioxidant capability and lower oxidative stress-related chronic diseases (Aruoma, 1998; Kohen and Nyska, 2002). *Clinacanthus nutans* is an example of a traditional herb that is rich in polyphenols and has potent antioxidant capabilities (Aromdee *et al.*, 2007; Yong *et al.*, 2013). It is traditionally used to treat oxidative stress-related diseases, such as diabetes and various kinds of cancers (Ching *et al.*, 2013; P'ng *et al.*, 2013).

The antioxidants protect cellular components by acting as radical scavengers, hydrogen and electron donors, peroxide decomposer, singlet oxygen quencher, enzyme inhibitors, synergist, and metal-chelating agents or by gene expression regulation (Frei *et al.*, 1988; Lobo *et al.*, 2010). The antioxidant process has been proposed to have two principle mechanisms of action, a chain-breaking mechanism and a prevention mechanism. The first mechanism donates an electron

from the primary antioxidant to the free radical. The free radical is unstable and highly reactive. Therefore, it either releases or steals an electron from another molecule in order to stabilise itself. This activity causes the molecule to become unstable, thus forming a second radical. The second radical then releases or steals an electron from another molecule, thus forming the following radical. This process is continued until a chain-breaking antioxidant helps to stabilise the free radical. On the other hand, the second mechanism involves the removal of ROS/RNS initiators by inhibiting chain-initiation catalyst in the cells (Pham-Huy *et al.*, 2008; Lobo *et al.*, 2010). Both mechanisms play a significant role in preventing oxidative stress-related diseases.

11. The defences against free radical attack and oxidative stress

Human body is naturally adapted with several defence mechanisms to counterbalance the excessive free radicals produced through the oxidative phosphorylation. Enzymatic antioxidant mechanism is the most important defence system against free radical. One such major free radical scavenging enzyme is the superoxide dismutase (SOD). Manganese SOD (MnSOD) was reported to be directly involved in the suspension of superoxide anions which was evident by the death of *sod2* gene-knocked out mice within 10 days after birth due to dilated cardiomyopathy, lipid amassing in liver and skeletal muscle and metabolic acidosis caused by the deficiency of MnSOD (Li *et al.*, 1995). Furthermore, tissues of those mice lacking MnSOD exhibited an elevated degree of oxidative damage DNA with higher rate cancer prevalence (Remmen *et al.*, 2003). Another type of SOD is the Copper, Zinc SOD (CuZnSOD) which is mainly localized in cytoplasm, nucleus, lysosome and intermembrane space of mitochondria (Chang *et al.*, 1988; Keller *et al.*, 1991; Crapo *et al.*, 1992; Sturtz *et al.*, 2001). Based on the study conducted

by Yim *et al.* (1990), CuZnSOD catalyzes hydroxyl radical ($\text{OH}\bullet$) from H_2O_2 . Deletion of *sod1* gene in knock-out mice was reported to show high level of oxidative damage to proteins, lipids, and DNA in skeletal muscle tissue due to lack of CuZnSOD (Muller *et al.*, 2006). Comprehensive oxidative damage was also observed in liver tissues of *sod1*^{-/-} mice (Elchuri *et al.*, 2005). Hence, CuZnSOD is a potential antioxidant enzyme.

Another enzymatic antioxidant defence mechanism is glutathione peroxidase (GSHPx) activity where free radicals are reduced by glutathione into water and its analogous alcohols (Wendel, 1980). The selenium-containing GSHPx is mainly localized in cytoplasm and mitochondria (Zarowski and Tappel, 1978; Epp *et al.*, 1983; Tappel, 1984; Timcenko-Youssef, 1985). The free radical defence mechanism through GSHPx is evident through the study conducted by Hawker *et al.* (1993) who demonstrated an excessive ROS generation in selenium-deficient mice. Although GSHPx-1 was reported to be insignificantly contribute against oxidative damage by evaluating carbonyl content, lipid peroxidation activity and rate of extracellular H_2O_2 consumption in several tissues of *GPx1*^{-/-} knock-out mice samples (Ho *et al.*, 1997), studies conducted by Zhang *et al.* (2009) revealed an elevation of free radicals in *Sod2*^{+/-} *Gpx1*^{-/-} mice through evaluation of DNA and protein oxidation in several tissues of the transgenic mice. In addition, *Gpx4*^{+/-} mice was reported to be highly sensitive to oxidative stress (Yant *et al.*, 2003; Ran *et al.*, 2003).

Furthermore, catalase is also a potential free radical defence enzyme which eliminates hydrogen peroxide by catalysing it into water and oxygen in which one molecule of catalase decomposes approximately six million of H_2O_2 molecules per minute (Kellin and Hartree, 1938; Mates *et al.*, 1999). Several studies have demonstrated the defence mechanism of free radicals by catalyse. Transgenic mice over expressing catalase (*hCatTg*^{+/0}) was

shown to decrease the generation of H_2O_2 which eventually resulted in reduced blood pressure in contrast to the wild-type mice (Yang *et al.*, 2002). In addition, knockout mice with under expression of catalase by disruption of *cat* or *cas1* gene was reported to show decreased rate of decomposition of hydrogen peroxide with an increased tendency to oxidative stress (Ho *et al.*, 2004).

On the other hand, there are several other non-enzymatic defence mechanisms against free radicals such as vitamin E, vitamin C and Thiol antioxidants. Vitamin E is a fat soluble vitamin which was reported to act against lipid peroxidation (Pryor, 2000). Vitamin C is a water soluble vitamin which acts in correspondence with vitamin E. α -tocopherol is an active form of vitamin E which eliminates lipid peroxyl radical by donating a hydrogen atom and itself becoming an α -tocopherol radical while vitamin C reduces α -tocopherol radical into its original form (Carr and Frei, 1999; Kojo, 2004). Thiol antioxidants include the tripeptide glutathione (GSH), thioredoxin (TRX), and α -lipoic acid (ALA) also acts against free radical through redox reactions (Rahman, 2007).

12. Medicinal plants as green approach defences against free radical attack

In recent times, substantial evidence emerged indicating a mutual relationship between the consumption of antioxidant-rich foods and the episodes of human health deterioration (Sies, 1997). Conversely, when synthetic antioxidants namely butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) were extensively employed as additives in food manufacturing business, a multitude of cases were reported on liver injuries and carcinogenesis (Grice, 1988; Wichi, 1986). Considering this fact, attentions were shifted into utilizing natural antioxidants. Plants, given the fact that can grow successfully adapting to the exposure of ultraviolet, are also reservoir for potent

antioxidants that quench free radicals, a physiological response usually not present in lower elevation plants (Alonso-Amelot, 2008).

12.1. *Verbascum sinaiticum* Benth. (Scrophulariaceae)

The plant, *Verbascum sinaiticum* is a biennial rosette plant (a taproot with a cluster of leaves on the soil surface), upon where this species is listed as rare in Egypt and wide-reaching to Sinai Peninsula (Hegazy, 2000). The leaves and flowers from *Verbascum* species had been utilized in the treatment of bronchitis, dry coughs, tuberculosis and asthma while studies have confirmed expectorant, mucolytic and demulcent properties (Kozan *et al.*, 2011). Extraordinary results were obtained from *V. sinaiticum* when it was observed for selectivity in anti-proliferation response against carcinoma and normal cells. The effect of anti-proliferate was evaluated in hepatocellular carcinoma (Hep-G2) and normal (MRC-5) cells after exposing the cells to the extract for 48 hours (Tauchen *et al.*, 2015). The extract comprises compositions of luteolin, chrysoeriol, hydrocarpin and sinaiticin which were earlier tested for cytotoxicity reaction towards leukaemia P-388 cells, providing a positive feedback (Afifi *et al.*, 1993). These compounds falls under the classification of flavonoids and flavonolignans (Afifi *et al.*, 1993 and Mahmoud *et al.*, 2007) to which they will not cause possible hazards to consumer and consequently an attribution to the selective anti-proliferative activity of *V. sinaiticum* against Hep-G2 and MRC-5 cell line (Tauchen *et al.*, 2015).

12.2. *Ugni myricoides* (Kunth) O.Berg

Ugni myricoides are inhabitants of western Latin America (WCSP, 2016), possessing the properties for anti-hypernociceptive effect (Quintão *et al.*, 2010) and anti-nociceptive effects in mice (Campêlo, 2011). Brovo *et al.*, had carried out an investigation between *U. my-*

ricoides fruits antioxidant properties and their inhibitory interaction against skin aging-related enzymes. *U. myricoides* has been measured for high values in mitigating free radical damages of UV light in skin, by both the ORAC (Oxygen radical absorbance capacity) and TEAC assays (The Trolox Equivalent Antioxidant Capacity). As a result, *U. myricoides* were determine to retain high total phenolic content, with high correlation coefficient above 0.80 and its aptitude in hindering collagenase, elastase, hyaluronidase and tyrosinase enzymes propose that the presence of high values of polyphenols are potential conducive for these responses (Bravo et al., 2016).

12.3. Black highland barley (BHLPE)

The black highland barley known in Tibet for a crop of high importance is referred as Qing Ke in Chinese. The potential of BHLPE was measured and the findings exhibited potent superoxide radical, hydroxyl radical and 2,2-diphenyl-1-picrylhydrazyl radical-scavenging activity, ferric reducing antioxidant power and moderate metal ion-chelating activity (Shen et al., 2016). Two groups of mice were prepared; a high fat diet (HFD) group and a group that has been administered with 600 mg BHLPE/kg body weight. The treated group of mice displayed remarkable reduction in total cholesterol, low-density lipoprotein cholesterol and the atherosclerosis index. The *in vivo* test further provides information on antioxidant defence system and antioxidant gene expression where significant amelioration had occurred within BHLPE treated group as compared to HFD mice group. Conclusively, the study highlighted the tendency of natural antioxidant of BHLPE in generating good health by reducing the incident of disease (Shen et al., 2016).

12.4. *Curcuma longa* L.

Curcuma longa L is a spice that has considerable usage in the practise of Ayurveda, Unani, Siddha, and Chinese

indigenous medicine for the administration of many ailments. It also has measurably multitude biological activities associated to antioxidant, anti-inflammatory and cancer preventive properties (Teiten et al., 2010; Goel et al., 2008; Hatcher et al., 2008).

In a paper reported by Dall'Acqua et al., a pattern of significant changes were observed in the urinary metabolome of healthy rats, orally treated with curcumin, compared to controls in data sets obtained both by NMR and HPLC-MS (Dall'Acqua et al., 2016). The effect of Curcuma extract on urinary composition in healthy rats disclosed evidence for *in vivo* reduction of the amount of urinary biomarkers of oxidative stress namely allantoin, 3-nitrotyrosine, m-tyrosine, 8-OHdG. The m-Tyrosine is examined as a biomarker for oxidative damage to proteins while urinary 8-OHdG (Orhan et al., 2004) is measured as a biomarker of comprehensive cellular oxidative stress due to its prevalent generation of oxidized DNA repair (Tsikas et al., 2005).

12.5. *Beta vulgaris* L.

Beetroot (*Beta vulgaris*) is well known for its high total phenolic content (50–60 $\mu\text{mol/g}$ dry weight) (Kähkönen et al., 1999; Vinson et al., 1998), and the presence of considerable amount of phenolic acids: ferulic, protocatechuic, vanillic, *p*-coumaric, *p*-hydroxybenzoic and syringic acids (Kujala, et al., 2000). It also a source of betalains, water-soluble nitrogenous pigments that feed on reactive oxygen species (ROS) to protect its plant from wound injuries and pathogenic penetration as studied in red beet (Sepúlveda-Jiménez et al., 2004). Five beetroot pomace extracts from different cultivar were studied upon where Detroit beetroot pomace extract (DBPE) exhibited the highest antiradical properties *in vitro*, by effectively scavenging DPPH radicals ($\text{EC}_{50}=2.06\pm0.10$ $\mu\text{g/ml}$) and high reducing power ($\text{EC}_{50}=123.39\pm06.05$ $\mu\text{g/ml}$). The effect of *in vivo* antioxidant and hepatoprotective activities indicated

reduction in the measurement levels of (xanthine oxidase, catalase-CAT, peroxidase, glutathione peroxidase-GSHPx, glutathione reductase, glutathione-GSH and thiobarbituric acid reactive substance with the administration of DBPE in doses of 2 and 3 ml/kg body weight (Vulic *et al.*, 2012; Vulic *et al.*, 2014).

12.6. *Syzygium aromaticum* L.

Clove (*Syzygium aromaticum*) is a spice regularly used in Asia and many parts of the world for cooking. The composition of *S. aromaticum* widens its properties ranging from antioxidant, anti-fungal, anti-viral, anti-microbial to anti-diabetic, anti-inflammatory, antithrombotic, anaesthetic, pain reliving and insect repellent properties (Parle and Khanna, 2011). Clove buds creates insulin-like interaction with hepatocytes and hepatoma cells by reducing phosphoenolpyruvate carboxykinase and glucose 6-phosphatase gene expression (Prasad *et al.*, 2005). It is a vital step to reduce postprandial hyperglycemia peak for the treatment of diabetes (Aguilar-Santamaría *et al.*, 2009). The presence of phenolic (free and bound) appears to hinder alpha-amylase and alpha-glucosidase in a dose-dependent manner. The extracts displayed high antioxidant activities as exemplified by their high reducing power, 1,1 diphenyl-2-picrylhydrazyl (DPPH) and 2, 2-azinobis-3-ethylbenzo-thiazoline-6-sulfonate (ABTS) radical scavenging abilities, as well as inhibition of Fe²⁺-induced lipid peroxidation in rat pancreas *in vitro* (Adefegha and Oboh, 2012).

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A Review on Green Synthesis of Nanoparticles and its Antimicrobial Properties

Karthika Arumugam^{1,*} and Naresh Kumar Sharma²

¹Department of Biotechnology, Kalasalingam University, Krishnankoil, Srivilliputtur, Tamil Nadu, India; ²Department of Biotechnology, Kalasalingam University, Krishnankoil, Srivilliputtur, Tamil Nadu, India; *Correspondence: karthika1386@gmail.com; Tel: +91-9489142440

Abstract: The most important aspect of nanotechnology is the synthesising and characterizing the nanoparticles (NPs). Nano particles are manufactured in large quantities because of their wide range of applications. All Physical, Chemical and Biological methods have been used in the metal nanoparticles production. The major problems in the production are the stability, aggregation, morphology, crystal growth, size and size distribution. In recent years, the green synthesis of metal NPs has become more attractive because of cost effectiveness and its various applications in developing new technologies. It is considered as an eco-friendly technology for the production of well characterized NPs. The metal nanoparticles produced by the plant are more stable and the rate of synthesis is faster as compared to other methods. Currently, researchers are mainly focused on searching new antimicrobial agents against various pathogenic bacteria which cause infectious diseases. Moreover, the NPs have effective antimicrobial properties against infectious pathogens. In this review, we have discussed different kind of plants which are used in synthesising NPs and highlighted their antimicrobial applications. We also discussed the basic mechanism by which nanoparticles interact with microbes.

Keywords: Antimicrobial activities; bacteria; green synthesis; nanoparticles; plants

1. Introduction

In modern science, nanotechnology is one of the most interesting areas of research. The synthesis of nano particle and nano materials is an important area of the nanotechnology. The size of synthesized nano particle could be in the range of 1 to 100 nm. Nowadays it has gaining a great importance in area such as cosmetics, health care, food and feed, biomedical, environment, mechanics, drug-gene delivery, health, optics, electronics, energy science, space industries, chemical industries, catalysis, light emitters, single electron transistors, nonlinear optical devices (Hoffman *et al.*, 1992) and photo-

electrochemicals (Colvin *et al.*, 1994; Wang and Herron *et al.*, 1991).

Additionally, tremendous application are increasing in the field of antimicrobials catalysis, microelectronics, bimolecular detection, diagnostics and therapeutics (Veera *et al.*, 2013) This is because of newly enhance physical, chemical and biological properties based on their size and morphology distribution. It was found that metallic nano particles are considered to have high antibacterial properties because of their large surface area. These nanoparticles find their application in the field of nanocomposites, medical imaging, filters, hyperthermia of tumors and drug delivery (Tan *et al.*, 2006; Panigrahi *et al.*, 2004). The antibac-

terial efficiency of nanoparticles made researcher to find the resistant strains against metal ions, antibiotics (Khalil *et al.*, 2013). Since there are the many approaches are available for the synthesis of nano particle like photochemical, thermal decomposition, microwave decomposition (Akl *et al.*, 2012).

Comparing all this, the best one is the eco-friendly way of green synthesis approach for the production of nanoparticles. This can be done by using plant extract, fungi, bacteria and enzyme. As there is the lack of chemicals this green synthesized nanoparticles have numerous benefits in the field of pharmaceutical genomics, immune response enhancement, biosensors, clinical chemistry and other biomedical applications (Diva *et al.*, 2012). These biosynthesized nanoparticles were found to be highly toxic against human pathogen which varies from simple prokaryotes to complex eukaryotes. The development of natural nano factories will depend on the ability of organism in the production of metal nano particles (Korbekandi *et al.*, 2009). The main aspects of producing highly stable and well characterized nano particle is achieved by selecting best organism and providing best optimal condition for the growth and enzyme activity.

Instead of using organism for synthesis of nanoparticles, plant extracts is consider to be cost effective and therefore can be used as an economic and best alternative for the large-scale production of metal nanoparticles. The biomolecules found in the plant extract will help in the bioreduction of metal nano particles in an eco-friendly way. Several plants are act as a source for green synthesizing nano particles. In present study the microbial routes and the plant extract that are used for synthesising nanoparticles were reviewed. And also the antibacterial mechanism of the NPs was discussed.

2. Green synthesis

Green synthesis is a green biologically based method of synthesising nano particles using microorganisms and plants in a cost effective, and an environment-friendly manner (Gowramma *et al.*, 2015; Makarov *et al.*, 2014). In Green synthesis method nano particle can be synthesized by utilizing (a) microorganisms like fungi, yeasts (eukaryotes), bacteria and actinomycetes (prokaryotes), (b) plants and plant extracts (c) templates like membranes, viruses DNA and diatoms. Micro-organism and plants are very stable to absorb and accumulate inorganic metallic ions and heavy metals from their surrounding environment (Beveridge and Murray, 1980; Singh *et al.*, 2011).

During synthesising nano particles using microorganism, optimisation of culturing medium, light, pH and temperature are very important. Thereby significantly increase enzyme activity (Iravani, 2011; Mukherjee *et al.*, 2001). According to Mittal *et al.* (2013) biosynthesis of nanoparticles using plants or plant based extracts are biologically safe and cost effective. Moreover nano particle synthesis using plant extract perform both reducing and stabilizing (capping) agents (Singh *et al.*, 2010; Sathishkumar *et al.*, 2009a).

3. Nano particle synthesized using plant

3.1. Gold nanoparticle

Gold nano particle act as a good source of green chemistry based techniques. The flower extract of *Nyctanthes arbortristis* (night jasmine) are used to synthesis spherical shaped Gold nanoparticle (Das *et al.*, 2011). *Coriandrum sativum* (coriander) leaf extracts are used to produce Au nanoparticles at different shape ranging in size from 7 to 58 nm (Narayanan and Sakthivel, 2008) According to Poinern *et al.* (2013) *Eucalyptus macrocarpa* leaf extract could be utilised to synthesize Au nanoparticles as well as silver nanoparticle with in a size from 50 to 200 nm. Moreover variety of plants sources such as the leaves and bark of *Fi-*

cus carica (Singh and Bhakat, 2012), *Sphaeranthus amaranthoides* (Nellore et al., 2012) and *Putranjiva roxburghii* (Badole and Dighe, 2012), plant extract of Mango (Yang et al., 2014); *Gymnocladus assamicus* (Tamuly et al., 2013); *Cacumen platycladi* (Wu et al., 2013); *Pogestemon benghalensis* (Paul et al., 2015); *Nerium oleander* (Tahir et al., 2015); *Butea monosperma* (Patra et al., 2015); Pea nut (Raju et al., 2014); *Hibiscus cannabinus* (Bindhu et al., 2014); *Sesbania grandiflora* (Das and Velusamy, 2014).

3.2. Silver nanoparticle

Normally silver (Ag) nanoparticles ranged in size from 15 to 65 nm with an average size of 34 nm and cuboidal, rectangular in shape. The medicinally important plants like *Boerhaavia diffusa* (Vijaykumar et al., 2014), *Aloe vera* (Chandran et al., 2006), *Terminalia chebula* (Edison and Sethuraman 2012), *Catharanthus roseus* (Mukunthan et al., 2011), *Ocimum tenuiflorum* (Patil et al., 2012) *Azadirachta indica* (Tripathi et al., 2009), *Emblica officinalis* (Ankamwar et al., 2005) *Cocos nucifera* (Roopan et al., 2013), common spices *Piper nigrum* (Shukla et al., 2010), *Cinnamomum zeylanicum* (Satishkumar et al., 2009a) have also been used for Ag NP's synthesis.

Au-Ag bimetallic nanoparticles also synthesize successfully by plants include *Azadirachta indica* (neem) (Shankar et al., 2004), *Anacardium occidentale* (cashew nut) (Sheny et al., 2011), *Swietenia mahagony* (West Indies mahogany) (Mondal et al., 2011) and cruciferous vegetable extracts (Jacob et al., 2012).

3.3. Copper nanoparticles

Many variety of plant extracts have been used for the synthesize of Copper (Cu) and copper oxide (CuO) nanoparticles. *Magnolia* leaf extract and *Syzygium aromaticum* (Clove) extracts is used to Cu nanoparticles with the size ranging from 40 to 100 nm (Subhankari and Nayak, 2013). Cu nanoparticles can

be synthesised using stem latex of *Euphorbia nivulia* (Common milk hedge). Some green methods for synthesis of copper nanoparticles are reported using plant leaf extracts such as *Capparis zeylanica* Linn (Saranyaadevi et al., 2014), tamarind, lemon juice (Sastry et al., 2013); *Ocimum sanctum* as capping agents (Kulkarni and Kulkarni 2013); *Magnolia kobus* leaf extract (Lee et al., 2013); *Syzygium aromaticum* (cloves) aqueous extract (Subhankari et al., 2013) and *Nerium oleander* (Gopinath et al., 2014). *Brassica juncea*, *Medicago sativa* and *Helianthus annuus* and *Tridax procumbens* (Abdul and Samarrai, 2012; Asim et al., 2012), *Lantana camara* (Majumder, 2012), *Zingiber officinale* (Ipsa and Nayak, 2013) and *Ocimum sanctum* (Vasudev and Pramod, 2013). Copper nanoparticles could be directly used for administration/*in vivo* delivery of nanoparticles for cancer therapy (Valodkar et al., 2011).

3.4. Copper oxide nanoparticles

According to Padile et al. (2013) *Sterculia urens* (Karaya gum) synthesizes Cuprous Oxide (CuO at a range of 4.8 nm size). Jayalakshmi and Yogamoorthi, (2014) tried out the copper oxide nanoparticles synthesis using flower extract of *Cassia alata*. Rinkesh et al. (2016) use Floral extract of *Caesalpinia pulcherrima* for the synthesis of CuO in an eco-friendly method.

3.5. Palladium nanoparticles

Palladium nanoparticles were synthesised using an extract of *C. Zeylanicum* taken from (cinnamon) bark Satishkumar et al. (2009b) and also using *Annona squamosa* (Custard apple) peel extract with the size ranging from 75 to 85 nm (Roopan et al., 2011). The leaf extract of soybean (*Glycine max*) have been able to synthesise nanoparticles with a mean size of 15 nm (Kumar et al., 2012). According to Nadagouda et al. (2008) *Coffea arabica* (Coffee) and *Camellia sinensis* (Tea) extracts have been utilised to synthesise

palladium nanoparticles at 20 to 60 nm in size. *Gardenia jasminoides* (Cape jasmine) act as a good source for synthesis of NPs. Many plant leaf extract are used for the synthesis of palladium nano particle such as *Pulicaria glutinosa* (Mujeeb *et al.*, 2014); *Anogeissus latifolia* (Kora *et al.*, 2015); *Cinnamomum camphora* (Yang *et al.*, 2010); *Curcuma longa* (Sathishkumar *et al.*, 2009c); *Gardenia jasminoides* (Jia *et al.*, 2009); *Glycine max* (Petla *et al.*, 2012); *Musa paradisiaca* (Bankar *et al.*, 2010); *Pinus resinosa* (Coccia *et al.*, 2012); *Pulicaria glutinosa* (Khan *et al.*, 2014) and *Moringa oleifera* (Anand *et al.*, 2016) have been reported.

3.6. Platinum nanoparticles

Song *et al.* (2010) was first reported the leaf extract of *Diospyros kaki* (Persimmon) were utilized for the synthesis of platinum nanoparticles. In this 90% of platinum ions were converted into 10% of leaf biomass at 95° C within the range of 2 to 12 nm. Platinum nano particle can also synthesized by using *Ocimum sanctum* (Holy basil) leaf extract within a range of 23 nm at 100° C (Soundarrajan *et al.*, 2012). Recently, very few reports available for the synthesis of Pt NPs using several plant extracts including *Cacumen platycladi*, *Prunus yedoensis*, *Azadirachta indica*, *Cochlospermum gossypium*, honey (Zheng *et al.*, 2013; Velmurugan *et al.*, 2016; Thirumurugan *et al.*, 2016; Vinod *et al.*, 2011; Venu *et al.*, 2011). Similarly *Diopyros kaki* plant (Jae *et al.*, 2010); *Lantana camara* (Musthafa *et al.*, 2016); *Quercus glauca* (Qg); *Azadirachta indica* (Thirumurugan *et al.*, 2016); *Ocimum sanctum* (Soundarrajan *et al.*, 2012); *Pinus resinosa* (Manikandan *et al.*, 2016).

3.7. Titanium dioxide nanoparticles

According to Roopan *et al.* (2012) TiO₂ nanoparticles was effectively synthesize by *Annona squamosa* peel. Sundrarajan and Gowri (2011) found that spherical sized titanium oxide with a range of 100 to 150 nm was synthesized by utilizing *Nyctanthes arbor-tristis* leaf.

Eclipta prostrata leaf extracts can able to produce titanium particles ranging in size from 36 to 68 nm (Rajakumar *et al.*, 2012; Zahir *et al.*, 2015). TiO₂ nanoparticles was found to be biologically synthesized by using *Catharanthus roseus* leaf extract ranged in size from 25 up to 110 nm Velayutham *et al.* (2011). Various plants are used for the synthesis of TiO₂ such as *Psidium guajava* (Thirunavukkarasu *et al.*, 2014); *Aloe Vera* plant extract (Ganapathi *et al.*, 2015); *Nyctanthes*, *Annona squamosa* peel extract, (Roopan, 2012) and *Eclipta prostrata* (Gong *et al.*, 2007), *Azadirachta indica* (Siegel *et al.*, 1999); *Azadirachta indica* (Anbalagan *et al.*, 2015).

3.8. Zinc oxide nanoparticles

ZnO nanoparticles have been synthesized using *Aloe vera* extract (Sangeetha *et al.*, 2011) in spherical shape. In addition, *Physalis alkekengi* extract was used to synthesis crystalline poly-dispersed. ZnO nanoparticles with size range of 72.5 nm (Qu *et al.*, 2011a) and were pseudo-spherical shape and with a size of 53.7 nm from *Sedum alfredii* (Qu *et al.*, 2011b). Pragati *et al.* (2016) was first to report the synthesis of zinc oxide nanoparticles using flower extract of *Nyctanthes arbor-tristis*. *Mimosa pudica* leaves extract and coffee powder extract were utilized for the synthesis of ZnO nano particle. Various plants are used for the synthesis of ZnO nanoparticles such as *Solanum nigrum* (Ramesh *et al.*, 2015); *Ocimum Tenuiflorum* (Sagar *et al.*, 2015); *Hibiscus subdariffa* (Niranjan *et al.*, 2015); *Cassia fistula* (Vidya *et al.*, 2013); *Agathosma betulina* (Thema *et al.*, 2015).

3.9. Indium oxide nanoparticles

Indium Oxide (In₂O₃) nanoparticles were synthesized by utilizing the leaf extracts from *Aloe vera* (*Aloe barbadensis* Miller) in spherical shaped with the size range from 5 to 50 nm (Maensiri *et al.*, 2008). *Astragalus gummifer* (Katira Gum) is used for the synthesis of Indium oxide nano particle (Kanchana *et al.*, 2016).

3.10. Iron nanoparticles

Fe nanoparticles was biologically synthesized using aqueous extract of sorghum bran at room temperature (Njagi *et al.*, 2011). According to Pattanayak *et al.* (2013), *Azadirachta indica* (Neem) were utilized to synthesis Fe nanoparticles with the size range from 100 nm. Shah *et al.* (2014) able to utilize stem extract of *Euphorbia milii* and leaf extracts of *Cymbopogon citrates* to synthesise Fe nano particle with the range of 43-42 nm. Additionally Fe nanoparticle can also synthesized using *Euphorbia milii*, *Tridax procumbens*, *Tinospora cordifolia*, *Datura innoxia*, *Calotropis procera* and *Cymbopogon citratus* (lemon grass tea). Plant parts like Mango leaves, Clove buds, Black Tea, Green tea leaves, Coffee seeds, Rose leaves, Cumin seeds, Origano leaves, Thymol seeds and Curry leaves for synthesising Fe nano particle (Monalisa and Nayak, 2013). Iron nano particle in association with silver can be able to synthesis by utilizing aqueous sorghum extract (Eric *et al.*, 2011).

3.11. Iron oxide nanoparticles

Iron oxide was successfully synthesized by (Yen *et al.*, 2016) using Seaweed *Kappaphycus alvarezii*. Latha and Gowri, (2014) found that caricaya papaya leaves extract were able to synthesis Fe_3O_4 nanoparticles at room temperature. *Eucalyptus globulus* leaf extract was utilized for the synthesis of Iron oxide by adding the extract into the aqueous solution of Ferric chloride (Matheswaran, *et al.*, 2014). Makarov *et al.* (2014) reported that aqueous extract of monocotyledonous (*Hordeum vulgare*) and dicotyledonous (*Rumex acetosa*) were utilized for the synthesis of iron oxide with the size ranging from 10 to 40 nm. Iron oxide magnetic nanoparticles (Fe_3O_4 -MNPs) were synthesized using the aqueous extract of White tea (*Camelia sinensis*) (Sara and Mahnaz, 2016). Mahnaz *et al.* (2013) work focused on the development of a biosynthetic method for the production

of Fe_3O_4 -NPs using brown seaweed (*Sargassum muticum*) extract.

3.12. Lead nanoparticles

Spherical shape Pb nanoparticles with the size of 10 to 12 nm were able by utilizing the latex from *Jatropha curcas* by Joglekar *et al.* (2011). Delma *et al.* 2016 tried out the green synthesis of Lead in association with copper nanoparticles by utilizing *Zingiber officinale* stem extract.

3.13. Selenium nanoparticles

Recently, Sasidharan *et al.* (2014) were able to synthesise spherically shaped particles Selenium (Se) nanoparticles using the extracts taken from the peel of citrus reticulata to produce with a mean particle size of 70 nm. Similarly Garima *et al.* (2014) approach is to utilize dried *Vitis vinifera* (raisin) extracts for biosynthesize selenium nanoparticles (Se-Nps) using. Fenugreek seed is used to synthesis selenium nanoparticle (Ramamurthy *et al.*, 2013). Various plants are used for synthesis selenium nano particle such as *Vitis vinifera* (raisin) extracts (Sharma *et al.*, 2014); *Clausena dentata* (Sowndarya *et al.*, 2016); *Leucas lavandulifolia* (Kirupagaran *et al.*, 2016) and *Capsicum annum* (Shikuo *et al.*, 2007).

4. Antimicrobial properties

In the field of biotechnology, biomineralization, bioremediation and microbial corrosion, Metal microbes interaction plays an important role (Prabhu *et al.*, 2012). Researchers found that metal oxide nanoparticles have good antimicrobial activity against fungi, virus and bacteria. Antimicrobial NPs have nanosized carrier for efficient delivery of antibiotics. This can prove the effectiveness for treating infectious diseases (Huh and Kwon, 2011). The susceptibility or tolerance of bacteria against Np differs among gram +ve and gram -ve. The mechanisms of NP toxicity depend on composition, surface modification and intrinsic properties.

The ionic silver released by the silver nanoparticles inactivates bacterial enzymes by interacting with thiol group. It inhibits bacterial DNA replication and damage the bacterial cytoplasm membranes thereby depleting the level of ATP and inhibit DNA unwinding, which leads to cell death. (Parveen *et al.*, 2012). In cause of copper NPs Metallic and ionic forms of copper produce hydroxyl radicals that damage essential proteins and DNA thereby inhibit the growth (Wang *et al.*, 2011). In case of Au, oxidation of Au_3^+ and decarboxylation of citrate generate free radicals in the presence of light. This will damage the DNA and essential protein of bacterial growth (Santo *et al.*, 2008). The ZnO NPs induce frame shift mutation in the bacteria and cause cell death. TiO_2 NPs peroxidise the phospholipid component of the lipid membrane thereby disturb the cell respiration and cause cell death. The exact mechanism of action of CdS Nps is not known. But it has antibacterial activity against *E. Coli* (Sarita, 2010) Fe_2O_3 nanoparticles to interact closely with microbial membranes, damaging their structure and inactivate bacteria.

5. Concluding remarks

This review has summarized the recent research work in the field of green synthesis of nanoparticles using plants. Plants are able to reduce metal ions faster than fungi or bacteria. Hence it is evident that the metal nanoparticles produced by plants are more stable while compared with nanoparticles produced by microbes. The capacity of plants in reducing ions depends on the presence of polyphenols, enzymes, and other chelating agents (in the plants). This could have the critical effects on the amounts of nanoparticle production. Nanoparticles are also demonstrated to have interesting antimicrobial activity against toxic pathogens. Usually, antibacterial activities of NPs depend on its physicochemical properties and type of targeted bacteria. The synthe-

sised green NPs have a good potential as an antimicrobial agent against different microorganism. However, further research is required to tap the potential.

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Production of Secondary Metabolites Using a Biotechnological Approach

Produtur Chandramati Shankar^{1,*} and Senthilkumar Rajagopal²

¹Department of Biotechnology, Yogi Vemana University, Kadapa-516003, AP, India;

²Department of Biochemistry, Rayalaseema University, Kurnool-518002, AP, India;
email: senthilanal@yahoo.com; *Correspondence: pchandra20@gmail.com; Tel: +91-8562-225426

Abstract: Secondary metabolites produced by Medicinal plants are useful for the production of life saving drugs. The use of natural phytochemicals has its own advantage as it has no side effects. Because of sustainability, attention on *in vitro* plant materials as potential factories for secondary phytochemical products is increasing. More than 80% of the world's population relies on traditional medicine as it has negligible side effects for their primary health care needs. Annually, about 95% of the medicinal plants' used as a raw material is growing at the rate of more than 40%. However, majority of medicinal plants are collected from their wild habitats, especially from forests. Repeated use of these medicinal plants has resulted in depletion and extinction of many important medicinal plant species from their natural habitats. The other most common reason is poor regeneration capacity of plant under natural habitats due to improper environmental factors like weather conditions, seasonal change, soil erosion etc. Due to these reasons, the need of hour is to protect and conserve valuable important medicinal plants otherwise many of these plants will be lost from natural vegetation forever. In this article, biotechnological approaches such as plant cell culture and hairy root culture is described as alternative methods for the production of secondary metabolites. The advantages of these methods and enhancements of secondary metabolites production by different methods are also discussed.

Keywords: Biotransformation; cell suspension culture; hairy root culture; immobilization; medicinal plants

1. Introduction

Plants produce a wide variety of chemical molecules that play important roles in its development and its adaptation to the environment. These molecules based on their functions and role in plants development have been grouped into two types namely, primary metabolites such as carbohydrates, lipids and amino acids etc and secondary metabolites which are low molecular weight compounds which have no recognized role in the maintenance of fundamental life processes in the plants that synthesize them but are known to play a

major role in the adaptation of plants to their environment. Many higher plants are major sources of natural products used as pharmaceuticals, agrochemicals, flavor and fragrance ingredients, food additives, and pesticides (Balandrin and Klocke, 1988). The search for new plant-derived chemicals should thus be a priority in current and future efforts toward sustainable conservation and rational utilization of biodiversity (Phillipson, 1990). It is estimated that about 100,000 plant secondary metabolites or natural products have been identified.

Over one quarter of new drugs that have been approved in the last 30 years are based on a lead from a molecule from plant origin. Moreover, 9 of the top 20 selling drugs are derived from knowledge of plant secondary metabolites (Harvey, 2000; Tulp and Bohlin, 2002). Higher plants are rich source of bioactive constituents or phyto-pharmaceuticals used in pharmaceutical industry. Some of the plant derived natural products include drugs such as morphine, codeine, cocaine, quinine etc; anti-cancer Catharanthus alkaloids, belladonna alkaloids, colchicines, phytostigminine, pilocarpine, reserpine and steroids like diosgenin, digoxin and digitoxin. Many of these pharmaceuticals are still in use today and often no useful synthetic substitutes have been found that possess the same efficacy and pharmacological specificity. Currently one fourth of all prescribed pharmaceuticals in industrialized countries contain compounds that are directly or indirectly, via semi-synthesis, derived from plants. Studies on plant secondary metabolites have been increasing over the last 50 years.

There are three potential pathways for primary metabolism: the Embden Meyerhof-Parnas Pathway (EMP), the Entner-Doudoroff pathway, and the hexose monophosphate (HMP) pathway. Due to rapid deforestation and depletion of genetic stocks, various efforts were made to implement new methods for several plant species conservation and also yielding in high amounts of secondary metabolites, with photosynthetic efficiency, pest resistant and disease resistant plants. To overcome these problem new improved methods of plant tissue culture and transformation through molecular approaches presents an alternative option for multiplication, development and conservation of elite plant species. Biotechnological approaches such as plant tissue culture hold great promise for controlled production of useful secondary metabolites on demand. The current yield and productivity cannot fulfill the commercial goal of plant cell-based bioprocess for the production of

most secondary metabolites. The recent advances, new directions, and opportunities in plant cell-based processes are being critically examined. Such as biotransformation using an exogenous supply of biosynthetic precursors, genetic manipulation, and metabolic engineering may improve the accumulation of compounds. Use of biotic and a biotic elicitors, can also be used for triggering the formation of secondary metabolites. The possible use of plant cell cultures for the specific biotransformation of natural compounds has been demonstrated (Cheetham, 1995; Scragg, 1997; Krings and Berger, 1998; Ravishankar and Ramachandra Rao, 2000). In the search for alternatives for production of desirable medicinal compounds from plants, biotechnological approaches, specifically, plant tissue cultures, are found to have potential as a supplement to traditional agriculture and also in the industrial production of bioactive plant metabolites (Ramachandra Rao and Ravishankar, 2000). Cell suspension culture systems could be used for large scale culturing of plant cells from which secondary metabolites could be extracted. Due to these advances, research in the area of tissue culture technology for production of plant chemicals has bloomed beyond expectations. The oncogenic strains of *Agrobacterium rhizogenes* is used to transform a range of plant species, which induces hairy roots. The hairy roots induced from *Agrobacterium rhizogenes* transformation has shown to have attractive properties for secondary metabolite production as compared to differential cell cultures (Kim *et al.*, 2002). While research to date has succeeded in producing a wide range of valuable secondary photochemical in unorganized callus or suspension cultures, in other cases production requires more differentiated micro plant or organ cultures (Davioud *et al.*, 1989). In this chapter we will be describing the popular types of biotechnological approaches for production of secondary metabolites.

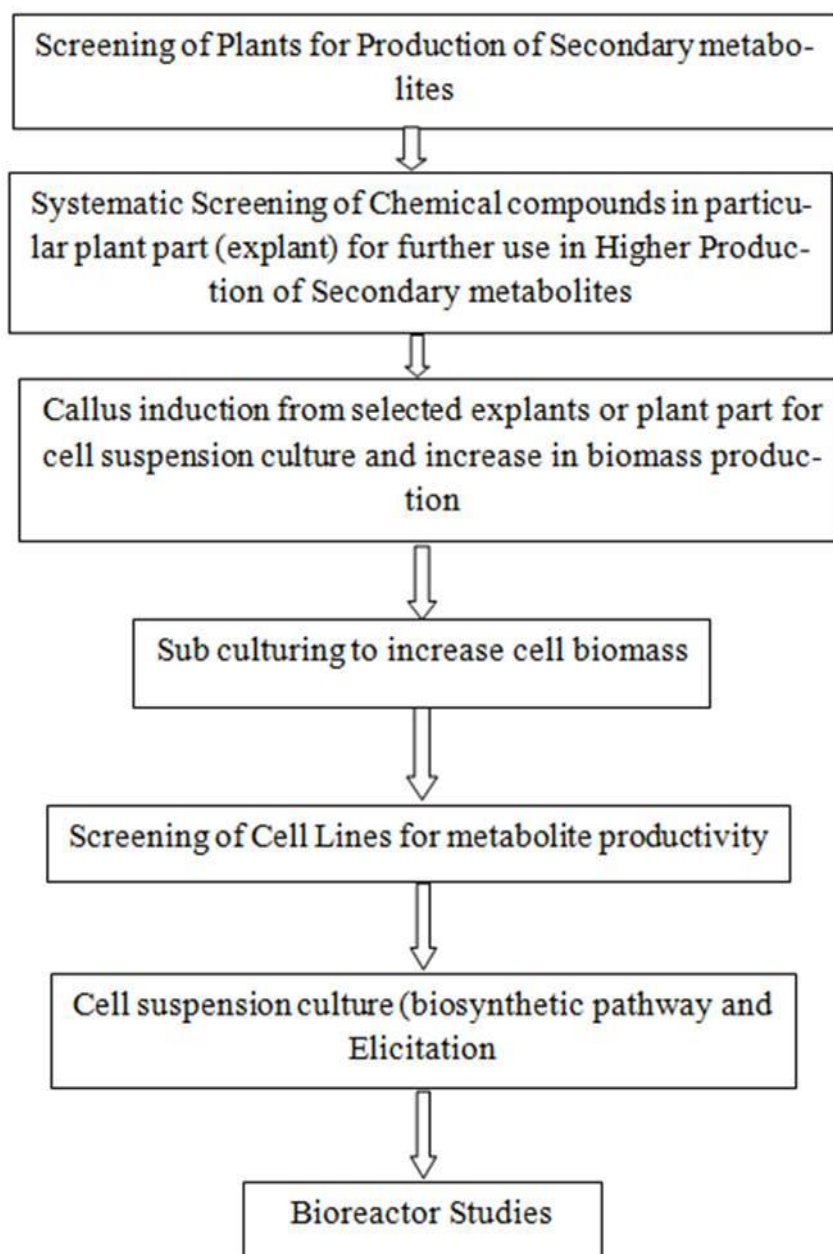


Figure 1: Schematic diagram of secondary metabolite production process.

2. Biotechnological approaches for production of secondary metabolites

The increased appeal of natural products for medicinal purposes coupled with the low product yields and supply concerns of plant harvesting has renewed interest in large-scale plant cell and hairy root culture technology. Secondary metabolites can be produced by using different biotechnological approaches (Table1). In this chapter, some techniques have been described.

2.1. Plant cell culture

Plant cell and tissue cultures can be established routinely under sterile conditions from explants, such as plant leaves, stems, roots, and meristems for multiplication and extraction of secondary metabolites. The explants selected from high yielding mother plants are usually selected as the starting material ((Figure 1)). Following the standard sterilization procedures the cultures are inoculated on a suitable plant tissue culture medium for induction of callus which is the base for all further production work (Figure 2). Callus cultures, can be divided into one of two

Table 1: Bioactive secondary metabolites produced using plant tissue cultures (Table courtesy: Vanisree and Tsay, 2004)

Plant name	Active ingredient	Culture type	Reference
<i>Agave amaniensis</i>	Saponins	Callus	Andrijany et al., 1999
<i>Ailanthus altissima</i>	Alkaloids	Suspension	Anderson et al., 1987
<i>Ailanthus altissima</i>	Canthinone alkaloids	Suspension	Anderson et al., 1986
<i>Allium sativum</i> L.	Alliin	callus	Malpathak and David, 1986
<i>Aloe saponaria</i>	Tetrahydroanthracene glucosides	suspension	Yagi et al., 1983
<i>Ambrosia tenuifolia</i>	Altamisine	Callus	Goleniowski and Trippi, 1999
<i>Anchusa officinalis</i>	Rosmarinic acid	Suspension	De-Eknamkul and Ellis, 1985
<i>Brucea javanica</i> (L.) Merr.	Canthinone alkaloids	Suspension	Liu et al., 1990
<i>Bupleurum falcatum</i>	Saikosaponins	Callus	Wang and Huang, 1982
<i>Bupleurum falcatum</i> L.	Saikosaponins	Root	Kusakari et al., 2000
<i>Camellia sinensis</i>	Theamine, γ -glutamyl derivatives	suspension	Orihara and Furuya, 1990
<i>Canavalia ensiformis</i>	L-Canavanine	Callus	Ramirez et al., 1992
<i>Capsicum annuum</i> L.	Capsaicin	Suspension	Johnson et al., 1990
<i>Cassia acutifolia</i>	Anthraquinones	Suspension	Nazif et al., 2000
<i>Catharanthus roseus</i>	Indole alkaloids	Suspension	Moreno et al., 1993
<i>Catharanthus roseus</i>	Catharanthine	Suspension	Zhao et al., 2001b
<i>Cephaelis ipecacuanha</i> A. Richard	Emetic alkaloids	Root	Teshima et al., 1988
<i>Chrysanthemum cinerariaefolium</i>	Pyrethrins	Callus	Rajasekaran et al., 1991
<i>Chrysanthemum cinerariaefolium</i>	Chrysanthemic acid and pyethrins	Suspension	Kueh et al., 1985
<i>Cinchona</i> L.	Alkaloids	Suspension	Koblitz et al., 1983
<i>Cinchona robusta</i>	Robustaquinones	Suspension	Schripsema et al., 1999
<i>Cinchona</i> spec.	Anthraquinones	Suspension	Wijnsma et al., 1985
<i>Cinchona succirubra</i>	Anthraquinones	Suspension	Khoury et al., 1986
<i>Citrus</i> sp.	Naringin, Limonin	Callus	Barthe et al., 1987
<i>Coffea arabica</i> L.	Caffeine	Callus	Waller et al., 1983
<i>Cruciata glabra</i>	Anthraquinones	Suspension	Dornenburg and Knorr, 1996
<i>Cryptolepis buchanani</i> Roem. & Shult	Cryptosin	Callus	Venkateswara et al., 1987
<i>Digitalis purpurea</i> L.	Cardenolides	Suspension	Hagimori et al., 1982
<i>Dioscorea deltoidea</i>	Diosgenin	Suspension	Heble and Staba, 1980
<i>Dioscorea doryophora</i> Hance	Diosgenin	Suspension	Huang et al., 1993
<i>Duboisia leichhardtii</i>	Tropane alkaloids	Callus	Yamada and Endo, 1984
<i>Ephedra</i> spp.	L- Ephedrine, D Pseudoephedrin	Suspension	O'Dowd et al., 1993
<i>Eriobotrya japonica</i>	Triterpenes	Callus	Taniguchi et al., 2002
<i>Eucalyptus tereticornis</i> SM.	Sterols and Phenolic compounds	callus	Venkateswara et al., 1986
<i>Fumaria capreolata</i>	Isoquinoline alkaloids	Suspension	Tanahashi and Zenk, 1985

Table 1: Continued...

Plant name	Active ingredient	Culture type	Reference
<i>Gentiana</i> sp.	Secoiridoid glucosides	Callus	Skrzypczak et al., 1993
<i>Ginkgo biloba</i>	Ginkgolide A	Suspension	Carrier et al., 1991
<i>Glehnia littoralis</i>	Furanocoumarin	Suspension	Kitamura et al., 1998
<i>Glycyrrhiza echinata</i>	Flavanoids	Callus	Ayabe et al., 1986
<i>Glycyrrhiza glabra</i> var. <i>glandulifera</i>	Triterpenes	callus	Ayabe et al., 1990
<i>Hyoscyamus niger</i>	Tropane alkaloids	Callus	Yamada and Hashimoto, 1982
<i>Isoplexis isabellina</i>	Anthraquinones	Suspension	Arrebola et al., 1999
<i>Linum flavum</i> L.	5-Methoxypodophyllotoxin	Suspension	Uden et., al 1990
<i>Lithospermum erythrorhizon</i>	Shikonin derivatives	Suspension	Fujita et al., 1981
<i>Lithospermum erythrorhizon</i>	Shikonin derivatives	Suspension	Fukui et al., 1990
<i>Lycium chinense</i>	Cerebroside	Suspension	Jang et al., 1998
<i>Mentha arvensis</i>	Terpenoid	Shoot	Phatak and Heble, 2002
<i>Morinda citrifolia</i>	Anthraquinones	Suspension	Zenk et al., 1975
<i>Morinda citrifolia</i>	Anthraquinones	Suspension	Bassetti et al., 1995
<i>Mucuna pruriens</i>	L-DOPA	Suspension	Wichers et al., 1993
<i>Mucuna pruriens</i>	L-DOPA	Callus	Brain, 1976
<i>Nandina domestica</i>	Alkaloids	Callus	Ikuta and Itokawa, 1988
<i>Nicotiana rustica</i>	Alkaloids	Callus	Tabata and Hiraoka, 1976
<i>Nicotiana tabacum</i> L.	Nicotine	Suspension	Mantell et al., 1983
<i>Ophiorrhiza pumila</i>	Camptothecin related alkaloids	Callus	Kitajima et al., 1998
<i>Panax ginseng</i>	Saponins and Sapogenins	Callus	Furuya et al., 1973
<i>Panax notoginseng</i>	Ginsenosides	Suspension	Zhong and Zhu, 1995
<i>Papaver bracteatum</i>	Thebaine	Callus	Day et al., 1986
<i>Papaver somniferum</i> L.	Alkaloids	Callus	Furuya et al., 1972
<i>Papaver somniferum</i>	Morphine, Codeine	Suspension	Siah and Doran, 1991
<i>Peganum harmala</i> L.	β -Carboline alkaloids	Suspension	Sasse et al., 1982
<i>Phytolacca americana</i>	Betacyanin	Suspension	Sakuta et al., 1987
<i>Picrasma quassioides</i> Bennett	Quassin	Suspension	Scragg and Allan, 1986
<i>Podophyllum hexandrum</i> royle	Podophyllotoxin	Suspension	Uden et al., 1989
<i>Polygala amarella</i>	Saponins	Callus	Desbene et al., 1999
<i>Polygonum hydropiper</i>	Flavanoids	Suspension	Nakao et al., 1999
<i>Portulaca grandiflora</i>	Betacyanin	Callus	Schroder and Bohm, 1984
<i>Ptelea trifoliata</i> L.	Dihydrofuro [2,3-b] quinolinium	Callus	Petit-Paly et al., 1987
<i>Rauwolfia sellowii</i>	Alkaloids	Suspension	Rech et al., 1998
<i>Rauwolfia serpentina</i> Benth.	Reserpine	Suspension	Yamamoto and Yamada, 1986
<i>Rauwolfia serpentina</i> x <i>Rhazya stricta</i>	3-Oxo-rhazinilam	Callus	Gerasimenko et al., 2001
<i>Rhus javanica</i>	Gallotannins	Root	Taniguchi et al., 2000
<i>Ruta</i> sp.	Acridone and Furoquinoline alkaloids and coumarins	Callus	Baumert et al., 1992

Table 1: Continued...

Plant name	Active ingredient	Culture type	Reference
<i>Salvia miltiorrhiza</i>	Lithospermic acid B and rosmarinic acid	Callus	Morimoto et al., 1994
<i>Salvia miltiorrhiza</i>	Cryptotanshinone	Suspension	Miyasaka et al., 1989
<i>Scopolia parviflora</i>	Alkaloids	Callus	Tabata et al., 1972
<i>Scutellaria columnae</i>	Phenolics	Callus	Stojakowska and Kisiel, 1999
<i>Solanum chrysotrichum</i> (Schldl.)	Spirostanol saponin	Suspension	Villarreal et al., 1997
<i>Solanum laciniatum</i> Ait	Solasodine	Suspension	Chandler and Dodds, 1983a
<i>Silybum marianum</i>	Flavonolignan	Root	Alikaridis et al., 2000
<i>Solanum paludosum</i>	Solamargine	Suspension	Badaoui et al., 1996
<i>Tabernaemontana divaricata</i>	Alkaloids	Suspension	Sierra et al., 1992
<i>Taxus</i> spp.	Taxol	Suspension	Wu et al., 2001
<i>Taxus baccata</i>	Taxol baccatin III	Suspension	Cusido et al., 1999
<i>Thalictrum minus</i>	Berberin	Suspension	Kobayashi et al., 1987
<i>Thalictrum minus</i>	Berberin	Suspension	Nakagawa et al., 1986
<i>Torreya nucifera</i> var. <i>radicans</i>	Diterpenoids	Suspension	Orihara et al., 2002
<i>Trigonella foenumgraecum</i>	Saponins	Suspension	Brain and Williams, 1983
<i>Withania somnifera</i>	Withaferin A	Shoot	Ray and Jha, 2001

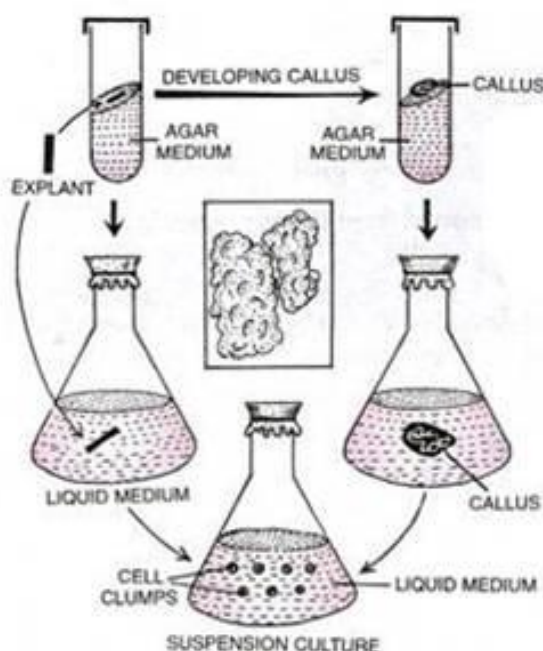


Figure 2: Schematic diagram showing the process of callus induction and cell suspension culture establishment.

types depending on the nature of callus i.e., compact or friable. In compact callus

the cells are densely aggregated, whereas in friable callus the cells are only loosely associated with each other and the callus become soft and breaks apart easily. Friable callus are a good source to establish a cell-suspension cultures. The friability of callus is improved by manipulating the medium components or by frequent sub-culturing or use of semi solid medium. When friable callus is placed into a suitable plant tissue culture liquid medium (usually the same composition as the solid medium used for the callus culture) and then agitated, single cells and/or small clumps of cells are released into the medium. Under the correct conditions, these released cells continue to grow and divide, eventually producing a cell-suspension culture. Use of large inoculum for initiation of cell suspensions cultures release more cell numbers into the medium quickly. Cell suspensions can be maintained just simply as batch cultures in conical flasks. They are continually cultured by repeated sub culturing into fresh medi-

um. This results in dilution of the suspension and the initiation of another batch growth cycle. The degree of dilution during subculture should be determined empirically for each culture. Too great a degree of dilution will result in a greatly extended lag period or, in extreme cases, death of the transferred cells. After subculture, the cells divide and the biomass of the culture increases in a characteristic fashion, until nutrients in the medium are exhausted and/or toxic byproducts build up to inhibitory levels this is called the 'stationary phase'. If cells are left in the stationary phase for too long, they will die and the culture will be lost. Therefore, cells should be transferred as they enter the stationary phase. It is therefore important that the batch growth-cycle parameters are determined for each cell-suspension culture. Strain improvement, methods for the selection of high-producing cell lines, and medium optimizations can lead to an enhancement in secondary metabolite production. Compared to whole plant cultivation system cell culture system has the advantages as follows:

- i. Useful compounds can be produced under controlled conditions independent of climatic change or soil conditions;
- ii. Cultured cells will be free of pathogens and pests;
- iii. Less space is required and production is uniform.

2.2. Hairy root culture

Agrobacterium rhizogenes a gram negative soil borne bacteria belonging to the family Rhizobiaceae, induces hairy root formation at the site of infection (Mugnier, 1988). Hairy roots are adventitious roots with lateral branching, growing rapidly and showing plagiotrophic growth with high branching and independent of plant hormones in the medium, these roots often possess the capacity to grow even when removed from the mother plant. The transformed roots generated show high differentiation and can cause stable and extensive production of secondary metabo-

lites. The secondary metabolites produced by hairy roots arising from the infection of plant material by *A. rhizogenes* are the same as those usually synthesized in intact parent roots, with similar or higher yields. This feature, together with genetic stability and generally rapid growth in simple media lacking phytohormones, makes them especially suitable for biochemical studies not easily undertaken with root cultures of an intact plant. The hairy roots are normally induced on aseptic, wounded parts of plants by inoculating them with *A. rhizogenes*.

2.3. Biotransformation using precursors

Commercial production of secondary metabolites requires a reproducible and standardized protocol for cultivation of plant cells/organs on a large scale. Employing precursor feeding, transformation methods, and immobilization techniques. The treatment of plant cells with biotic and/or abiotic elicitors has been a useful strategy to enhance secondary metabolite production in cell cultures (Karuppusamy, 2009). The most frequently used elicitors are fungal carbohydrates, yeast extract, Methyl Jasmonate (MJ) and chitosan. MJ, a proven signal compound, is the most effective elicitor of taxol production in *Taxus chinensis* Roxb (Wink *et al.*, 2008) and ginsenoside production in *P. ginseng* C.A. Meyer cell/organ culture (Xu *et al.*, 2008; Yamanaka *et al.*, 1996).

2.4. Immobilization of cells for secondary metabolite production

One of the major limiting factors in the development of a commercial production system using plant cell culture has been the production cost of phytopharmaceuticals. The use of high biomass levels for extended periods would be one method of increasing productivity and hence reducing the costs. This can be achieved by the immobilization of plant cells. Immobilization is the newest culture technology of plant cell, and considered as to be the most "natural". It has been defined as a technique, which confines to a catalytical-

ly active enzyme or to a cell within a reactor system and prevents its entry into the mobile phase, which carries the substrate and product. The first successful immobilization of plant cells was reported by Brodelius *et al.*, (1979) and they entrapped *Catharathus roseus* and *Daucus carota* cells in alginate beds. Following success with enzymatic and microbial process, immobilization has been suggested as a strategy to enhance the overall productivity of secondary metabolite in plant cell culture. The ability to immobilize plant cells has been reported for a large number of plant cells and protoplasts by using a variety of polymers. Immobilization of plant cells has been used for a wide range of reactions, which can be divided into three groups. (1) Biotransformation or bio-conversion, (2) synthesis from precursors and (3) the *De Novo* synthesis of compounds. Some of the advantages of immobilization are, retention of biomass enables its continuous reutilization as a production system. The immobilization of cells allows the use of a higher biomass level compared to cell suspension culture, Separation of cells from medium and the product is extra cellular, which will simplify downstream processing compared to extract from tissue. Immobilization allows a continuous process, which increase volumetric productivity and allows the removal of metabolic inhibitors. Reduces problems such as aggregate, growth and foaming. Some of the disadvantage is the microenvironment favoring optimal production can be unfavorable for released secondary metabolites and cause their degradation or metabolism.

2.4.1. Different types of immobilization

- i. Direct intracellular binding due to natural affinity (adsorption, adhesion and agglutination).
- ii. Covalent coupling on otherwise inert matrices.
- iii. Intracellular connection via bi or poly functional reagent (cross-linking).

- iv. Mixing with suitable materials, changing their consistency with temperature (embedding).
- v. Physical retention within the framework of diverse pore size and permeability (entrapment, micro encapsulation).

2.4.1.1. Selection of immobilization system

The choice of a suitable immobilization system is determined by the following requirements.

- i. The polymer material used for immobilization must be available in large quantities; it must be inert, non-toxic and cheap.
- ii. It must be able to carry large quantities of biomass and its fixing potential must be high.
- iii. The immobilization process must not diminish enzymatic activity of biological catalyst.
- iv. Manipulation of the biological catalyst must be as simple as possible.

2.4.2. Methods for immobilization

2.4.2.1. Gel entrapment by polymerization

A monomer or a mixture of monomers is polymerized in the presence of a cell suspension, which is entrapped inside the lattice of the polymer.

2.4.2.2. Gel entrapment by ionic net work formation

In this method, polymerization of polyelectrolyte is achieved by addition of multivalent ions. The most common method is the entrapment in calcium alginate. This is a non-toxic process in which sodium alginate solution containing the cell suspension is dropped into a mixture of counter ion solution such as calcium chloride. A uniform, spherical and highly microspores structure results, which retains the cell.

2.4.2.3. Gel entrapment formation by precipitation

Gels may be formed by precipitation of some natural and synthetic poly-

mers by changing one or more parameters in the solution, such as temperature, salinity or PH of solvent. Several materials can be used for entrapment. The examples include methods involving thermal treatment. Some disruption of viability can occur naturally.

2.4.2.4. Entrapment in preformed structures

Hollow fiber reactors can be used to immobilize plant cells by entrapment. The cells are placed on the shell side of the hollow fibre cartridge and nutrient medium is rapidly re-circulated through the fibers. This may have important applications in large-scale.

2.4.2.5. Surface immobilization

Surface immobilization may occur on both natural and other matrices. Examples of natural matrices are deeper callus layers and cellulose, while synthetic one includes nets of steel and nylon.

2.4.2.6. Immobilization by embedding

The temperature dependent solubility of macromolecules like agarose, agar and carrageenan or the differing solubility of the sodium and calcium salts in the case of alginate are utilized to form polymeric gels or gel combination. Insoluble are formed under cold conditions (Agar) or in aqueous CaCl_2 solutions (Alginate). Their structure is non-uniform, with differing pore diameters at the surface and in deeper layers. The size and form of the beds can be determined in part by stirring speed and using alginate, by the viscosity of the solution and dropping aperture.

2.4.2.7. Types of bioreactors used for immobilization of plant cells

The following types of reactors are generally used for immobilized plant cell: (1) Packed bed reactors (2) Well mixed reactor (3) Fluidized bed reactors (4) Membrane reactors

2.5. Metabolic engineering and production of secondary metabolites

Metabolic engineering involves the targeted and purposeful alteration of metabolic pathways found in an organism to achieve better understanding and use of cellular pathways for chemical transformation, energy transduction, and supramolecular assembly (Lessard, 1996). This technique applied to plants will permit endogenous biochemical pathways to be manipulated and results in the generation of transgenic crops in which the range, scope, or nature of a plant's existing natural products are modified to provide beneficial commercial, agronomic, and/or postharvest processing characteristics (Kinney, 1998).

Several genes in the biosynthetic pathways for scopolamine, nicotine, and berberine have been cloned, making the metabolic engineering of these alkaloids possible. Expression of two branching-point enzymes was engineered: putrescine *N*-methyltransferase (PMT) in transgenic plants of *Atropa belladonna* and *Nicotiana sylvestris* and (S)-scoulerine 9-*O*-methyltransferase (SMT) in cultured cells of *C. japonica* and *Eschscholzia californica*. Over expression of PMT increased the nicotine content in *N. sylvestris*, whereas suppression of endogenous PMT activity severely decreased the nicotine content and induced abnormal morphologies. Ectopic expression of SMT caused the accumulation of benzyloquinoline alkaloids in *E. californica* (Sato *et al.*, 2001).

3. Few commercial products obtained by tissue cultures

Some of the commercial products obtained by plant tissue culture are listed below:

3.1. Taxol

Taxol (paclitaxel), a complex diterpene alkaloid found in the bark of the *Taxus* tree, is an anticancer agent. At present; production of taxol by various *Taxus* species cells in cultures has been one of the most extensively explored areas of plant cell cultures.

3.2. Morphine and codeine

Latex from the opium poppy, *Papaver somniferum*, is a commercial source of the analgesics, morphine, and codeine. Callus and suspension cultures of *P. somniferum* are being investigated as an alternative means for the production of these compounds. Biotransformation of codeinone to codeine with immobilized cells of *P. somniferum* has been reported by Furuya *et al.*, (1972). The conversion yield was 70.4%, and about 88% of the codeine converted was excreted into the medium.

3.3. L-Dopa

L-3, 4-dihydroxyphenylalanine, is an important intermediate of secondary metabolism in higher plants and is known as a precursor of alkaloids, betalain, and melanine, isolated from *Vinca faba*, (Daxenbichler *et al.*, 1971) *Mucuna*, *Baptisia*, and *Lupinus* (Brain and Lockwood, 1976).

3.4. Diosgenin

Tal *et al.* (1983) reported on the use of cell cultures of *Dioscorea deltoidea* for the production of diosgenin. They found that carbon and nitrogen levels greatly influenced diosgenin accumulation in one cell line. Ishida (1988) established *Dioscorea* immobilized cell cultures, in which reticulated polyurethane foam was shown to stimulate diosgenin production, increasing the cellular concentration by 40% and total yield by 25%.

3.5. Capsaicin

Capsaicin, an alkaloid, is used mainly as a pungent food additive in formulated foods (Ravishankar *et al.*, 2003). It is obtained from fruits of green pepper (*Capsicum* spp.). Capsaicin is also used in pharmaceutical preparations as a digestive stimulant and for rheumatic disorders (Sharma *et al.*, 2008). Suspension cultures of *Capsicum frutescens* produce low levels of capsaicin, but immobilizing the cells in

reticulated polyurethane foam can increase production approximately 100-fold.

3.6. Camptothecin

Camptothecin, a potent antitumor alkaloid, was isolated from *Camptotheca acuminata* (Padmanabha *et al.*, 2006; Sakato and Misawa, 1974) induced *C. acuminata* callus on MS medium containing 0.2 mg/l 2, 4-D and 1 mg/l kinetin and developed liquid cultures in the presence of gibberellin, l-tryptophan, and conditioned medium, which yielded camptothecin at about 0.0025% on a dry weight basis. When the cultures were grown on MS medium containing 4 mg/l NAA, accumulation of camptothecin reached 0.998 mg/l (Thengane *et al.*, 2003).

3.7. Berberine

Berberine is an isoquinoline alkaloid found in the roots of *Coptis japonica* and cortex of *Phellodendron amurense*. This antibacterial alkaloid has been identified from a number of cell cultures, notably those of *C. japonica*, (Vanisree and Tsay, 2004; Breuling *et al.*, 1985; Sato *et al.*, 2001) *Thalictrum* spp., (Nakagawa *et al.*, 1984; Suzuki *et al.*, 1988) and *Berberis* spp. (Breuling *et al.*, 1985). The productivity of berberine was increased in cell cultures by optimizing the nutrients in the growth medium and the levels of phytohormones (Sato and Yamada 1984; Nakagawa *et al.*, 1986; Morimoto *et al.*, 1988). By selecting high-yielding cell lines, Mitsui group produced berberine on a large scale with a productivity of 1.4 g/l over 2 weeks. Other methods for increasing yields include elicitation of cultures with a yeast polysaccharide elicitor, which has been successful with a relatively low-producing *Thalictrum rugosum* culture (Funk *et al.*, 1987).

4. Concluding remarks

Industrial compounds obtained and prepared from plant sources are in high demand. This is because it is safer with no side effects and cheaper compared to syn-

thetic products. Many lead molecules in drugs are from plant origin; hence, large amount of natural resources are destroyed for the production of these compounds. The alternate means of production of industrially important compounds is needed for the sustainability of natural resources and preserve the diversity in the ecosystem. There are many advanced approaches which can be used for the production of secondary metabolites; but, only limited methods have been highlighted in this article. Biotechnology can play an important role in production of useful secondary metabolites and will help in sustained development of plant diversity.

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Potential of Marine Algae Derived Extracts as a Natural Biostimulant to Enhance Plant Growth and Crop Productivity

Lakkakula Satish and Manikandan Ramesh*

Department of Biotechnology, Science Campus, Alagappa University, Karaikudi, Tamil Nadu, India; *Correspondence: pandu.pine@gmail.com / mrbiotech.alu@gmail.com; Tel: +91 4565 225215

Abstract: Photobioreactor (PBR) is a reactor which exploits a light supply to farm phototrophic microorganisms which can generate biomass using light, water and carbon dioxide. Macro- and microalgae are developed naturally in fresh water as well as marine water (highly economical) or brackish water. Universal production of marine algal monocultures is basically limited to a selective species such as *Scenedesmus spp.*, *Nannochloropsis spp.*, and *Chlorella spp.*, and some extremophiles like *Arthrospira spp.*, and *Dunaliella spp.*. Owing to their inborn resistance to predators and competitors, algal species can be cultivated naturally in open systems akin to ponds, stirred tanks and bubble columns. Nowadays, numerous research groups are focusing on PBRs (viz., selective closed type or open systems) to produce marine algae (cyanobacteria or seaweeds) through utilizing a light source with lavish biomass accumulation. The cultivation of marine algae has distinguished a new extension in numerous fields crucial for humanity, especially energy, food, health and environment. Currently, marine algae and algae-based extracts remain predominantly unexploited despite their huge potential applications. This chapter highlights the present and future prospects of marine plants and their derived aqueous extracts for crop improvement and enhancement of plant biomass production.

Keywords: Crop protection; cyanobacteria; marine seaweed; photobioreactor; plant biostimulants; plant growth hormones

1. Introduction

As per previously published data about micro algae biotechnology, the foremost concern of external mass farming has been planned at obtaining successful consumption of prominent light potency (Masojidek *et al.*, 2003). The word algae cover a wide choice of diverse organisms which can be normally illustrated as eukaryotic protests (a complicated group to describe), that are diverse from plants but are naturally aquatic and photosynthetic. They can also be microscopic single celled micro algae or larger, more complex multi cellular seaweeds. They can be found all-inclusive in both

fresh water and marine environments across a wide range of habitats. Micro algae, comprise of cyanobacteria and seaweeds (macroalgae) are a resource of valuable bioactive compounds including vitamins, pigments, secondary metabolites, plant growth hormones and other food appurtenances representing extremely discriminatory pharmacological activities (Masojidek *et al.*, 2009; Satish *et al.*, 2015, 2016, Rency *et al.*, 2017). Marine algae (macro and micro) is the vital resource for bio-fuel production, since they can collect a large quantity of lipid content within their cells and contain very huge biomass productive capacity (Wu and Merchuk, 2004; Yang *et al.*, 2014). In

case of filamentous micro algae, a variety of strains are recognized to make intra- or extracellular metabolites by dissimilar biological activities (Glombitza and Koch, 1989). However, numerous research groups focusing on photobioreactors (PBRs) for making of prominent algal biomass production, is not nearly as superior as requisite by the worlds desires, particularly in the pharmaceutical, for human utilization as well as fish and animal feed, and for field level agricultural applications. Still, there is inert question as to whether algal based cultures are able to make plant growth stimulants and concerning their mechanisms of action towards elevated yielding, biotic and abiotic stress resistance and high biomass production of crops in agriculture through regulation of growth succession such as cell division and in the sensitivity of environmental changes.

2. Recent developments for cultivation of marine algae using photobioreactors

The majority of marine algae use photosynthesis to confine light energy to change inorganic substances into useful sugars and then other molecules which is similar to plants. The limitations of development forced by the constraint and saturation of light and results have impelled algal biotechnologists to come upon for resolution to convince this outcome in outdoor cultures (Masojidek *et al.*, 2003). Due to the significance of light saturation, information on the behavior of cyanobacteria cultures showed to very high illumination is comparatively sparse and most of it has been acquired in laboratory experiments. The possible development of algal biotechnology is moving towards the field of high charge commodities grown in well controlled cultivation conditions, such as temperature, nutrient supply, pH, light, and CO₂ (Masojidek *et al.*, 2009). Aquaculture has turned into extremely important in the salvation of food and nutrition for the growing world

population over the foregoing few decades. In the year 2012, an incredible 90.4 million tonnes of marine based food and beverages were farmed and this amount is expected to increase until about 2030, at which moment it is indefinite that capture fisheries and aquaculture will distribute equal quantities (FAO, 2013; Taelman *et al.*, 2015). Nevertheless, an entire control on culture conditions is sufficient only through closed systems, only PBR.

Commonly, PBR is a bioreactor which uses a light source to grow phototrophic microorganisms which can produce biomass through light and CO₂ and include plants, macro and micro algae, cyanobacteria, purple bacteria and mosses. The first step was taken by Richmond and group in 1978 as the introduction of external algal biotechnology in Israel (Richmond and Vonshak, 1978; Masojidek *et al.*, 2003, 2009). Compared to open culture systems like ponds and raceways, PBR systems have several advantages viz., reproducible farming with regard to environmental changes, maintenance of dissolved oxygen concentration, sufficient mixing of the culture with the appropriate flow rate, the opportunity of temperature regulation with low CO₂ losses and making it feasible to focus and regulate the irradiance for augmentation of algal cultures with reduced risk of culture contamination (Masojidek *et al.*, 2009). Rorrer *et al.* (2004) showed the bioprocess engineering method, especially for the marine algae process through the development of cell and tissue culture systems and his group described a diversity of techniques to grow phototropic suspension cultures appropriate for the cultivation in PBR systems. Also, the same group compared three foremost PBR configurations for macroalgal suspension culture systems viz., bubble-column or airlift, tubular recycle and stirred tank, and considered the major factors that limit their development and cultivation performance (Rorrer *et al.*, 2004). Later on, a novel tubular PBR with linear Fresnel lenses depends on solar concentrators dis-

covered and studied the correlation between changes in physiological and photochemical characters in central Europe (Masojidek *et al.*, 2009). Nevertheless, a number of guideline ideologies about the best possible design of PBRs have been established (refer for recent reviews, Pulz, 2001; Carvalho *et al.*, 2006). But, still the worldwide monoculture of algae is largely limited to some unique species, including extremophiles, easy and quick growers and this slow growing spp., have to be cultivated using PBR systems in order to certify their control, although the cultivation of subtle spp., needs unusual protection to keep away from forces which could give stress to the cells, centrifugal forces, mainly surface and shear stress (Masojidek *et al.*, 2009).

3. A long history of algal use

Over the past two decades a great covenant of literature has been focused on the PBR potential of algal commercial applications, due to space limitations we account a few of them in this chapter. Kelps are cost-effectively precious and primary producers, as a result, numerous studies on algal cultivation through breeding have considered enhancing its quality and productivity (Sato *et al.*, 2017). However, most cultivation tests have been performed in the ocean, thereby limiting the development of new cultivars. Macroalgae was being eaten at least 1,500 years ago in Japan and it remains an important food in many cultures where it is valued for its high mineral content (i.e., Nori and Laverbread). Closer to home in Europe, kelp was farmed extensively from the 17th to 19th centuries for processing into soda for the linen industry and into iodine for medicinal purposes. Micro algae have been used for decades as a food supplement (i.e., spirulina) and as a feedstock for farmed shellfish and finfish. Compounds extracted from both micro- and macroalgae today find their way into everyday foods, health, cosmetics and pharmaceutical industries, agricultural fields,

energy and environment (Muller-Feuga *et al.*, 2012). Greater attention to several enterprises and businesses has to be the reality that there is plenty of other value added product opportunities that exist based on algae. The recombinant pharmaceutical protein production began to be improved more than 25 years before and today above 300 protein commodities are in the market or in late clinical stages (Decker and Reski, 2008). Though, a few years back, the moss *Physcomitrella patens* were suggested and merchandised as substitute production host that convene this concern through providing a special amenability for accurate genetic engineering together with economic cultivation (Decker and Reski, 2008). However, the production cost of algae in large scale is not yet competitive, predominantly because of the prevalence of the PBR technology worldwide. As well the major progress in classic PBR design, a number of novel configurations have been anticipated in the last two decades to advance their management expressed in terms of biomass productivity, photosynthetic efficiency, light absorption and light to biomass acquiesce.

4. Advantages of PBRs in algal cultivation and exploring the power of algae

The novel advantages of PBR have been recommended in the last two decades as a substitute to the open type cultures and they are widely used in food, cosmetics and pharmaceutical industries to produce algal biomass in large scale. In another way, the cost of nutrient media for algal (cyanobacteria) cultivation is greatly cheaper than to heterotrophic bacteria and the inorganic nutrients at low concentration limit the contamination through other microorganisms (Chetsumon *et al.*, 1994). The harvesting cost can be controlled through the production of bioactive substances from the marine algae cultivated by PBRs. An evidence of the rising interest in PBR applications is

the large number of research and review articles published over the past few years showing novel PBR developments (Olivieri *et al.*, 2014; reviewed in this chapter). An interesting application of micro algal cultures in PBRs concerns the welfare of space mission teams. A PBR intended to produce algae protein along with oxygen and to remediate devastate and CO₂ can form a closed ecological life support system where it is elemental for astronauts concerned in long term investigation task and on space stations on other planets like the Moon and Mars (Olivieri *et al.*, 2014). Recently, Cao *et al.* (2012) patented a method for micro algal biomass cultivation by 100% CO₂ to prolong the life support system in such a harsh environment.

Algal products are again being commercially explored and developed with the help of a growing global industry using the latest algal biotechnologies. This involves the mass cultivation of micro- and macroalgae and conversion of the harvested biomass into a range of value-added products. The EnAlgae project aims to develop technologies that will be both economically-viable and environmentally-friendly ways so that the production of algal biomass can be rolled out on industrial scales. Since macroalgae generates energy through photosynthesis, algae biomasses are situated in the aquatic euphotic zone upper layers. Algal photosynthetic systems are comparable to that of plants growing on terrestrial lands, however, usually, they are highly capable of converting sunlight into biomass since less complex cellular structure and their direct access to water, nutrients and CO₂ (Kilinc *et al.*, 2013; Taelman *et al.*, 2015). Chetsumon *et al.* (1994) produced antibiotic through the immobilized cyanobacterium, *Scytonema* spp., in a seaweed type PBR. European Union imports of macroalgae have conventionally been used as the products for agriculture or in the food, pharmaceutical and cosmetic industries for their useful extracts and are very less frequently used for direct human

consumption (Ngo *et al.*, 2011; Taelman *et al.*, 2015).

5. Extraction technology of bioactive compounds from marine algae

Extraction technology is critical for the complete use of marine algae (seaweeds and cyanobacteria) raw materials to reducing time, with high yields at low costs and with low consumption of solvents (Caamal-Fuentes *et al.*, 2013). Natural bioactive composite consists of an extensive range of functionalities and structures which give an outstanding pool of molecules for the assembly of functional foods, nutraceuticals and food additives. A few of those natural compounds can be originated in nature at an elevated level eg., polyphenols but other compounds can only be available at very small concentrations, so that enormous harvesting is essential to acquire adequate amounts. The structural complexity of the compounds makes chemical synthesis unsuccessful and the intrinsic complications in producing and screening of these compounds include led to the improvement of advanced technologies (Gil-Chavez *et al.*, 2012). Marine algae derived bioactive compound production may be regulated by the assortment of suitable cultivation conditions and making these algae accurate natural bioreactors (Ibanez *et al.*, 2012). For extraction of novel compounds from algae, it is essential to estimate how efficient ingredients are acquired. In this view, there is a need to unite relevant, cost-effective, choosy and environmentally friendly extraction methods with the permitting requirements about the use of food grade reagents/solvents and progressions. The application of nature friendly, advanced and clean extraction methods allows for the accomplishment of the aimed compounds of significance with high efficient extraction techniques, whereas, at the similar moment in time, reducing the utilize of organic toxic solvents.

The “Green Chemistry” association has been investigating traditions to ease the risk of harmful chemical contact to the environment and humans. Ibanez *et al.* (2012) showed a schematic diagram which stated about the changes in the waste prevention hierarchy. The innovation and progress of marine algae based bioactive compounds is a quite new area when compared to the invention of bioactive compounds from other global resources. As a result, in the growth of this field, novel, eco-friendly and sustainable trends should be followed (reviewed by Ibanez *et al.*, 2012).

6. Application of algal extracts for plant growth and development

Worldwide, the policy drivers supporting the application of agricultural biostimulants derived marine algae in agriculture and are also emphasised in crop management through soil drenches, seed priming, hydroponic treatments and foliar sprays (Sharma *et al.*, 2014). The majority of the research works associated to crop improvement and protection has attracted in recent times an extraordinary awareness in order to widen safer and new control methods as an alternative of these methods depending on chemical pesticides. One approach might be the generation of the self-defenses in plants through natural extracts (Terry and Joyce, 2004; Walters *et al.*, 2005). Marine micro- and macroalgae are the abundant for several bioactive compounds, including algal polysaccharides, and the composition and structure of these compounds have greatly contributed to their potential on activating signaling pathways and enhancing defense mechanisms in a variety of plants (El Modafar *et al.*, 2012; Arman and Qader, 2012; Abouraicha *et al.*, 2015; de Freitas and Stadnik, 2015). In the late 1940's, the first seaweed liquid extract for agricultural utilize has been urbanized and sold as Maxicrop (Satish *et al.*, 2015). All through the past two decades, β -(1,4)-D-polyglucuronic acids (glucuronans)

synthesized from marine algae have been industriously illustrated in literature for their biological and physico-chemical characteristics in various models (Caillot *et al.*, 2012). To give an instance, a linear β -1,3-glucan polymer known as laminarin derived from brown algae provoked the development of antifungal compounds in alfalfa (*Medicago sativa*) cotyledons (Kobayashi *et al.*, 1993) and a number of defense responses in rice (*Oryza sativa*) (Inui *et al.*, 1997), tobacco (*Nicotiana tabacum*) cell suspension cultures (Klarzynski *et al.*, 2000), and grapevine (*Vitis vinifera*) (Aziz *et al.*, 2003). The effectiveness of two different algal saccharides, glucuronan and oligoglucuronans against postharvest gray mold caused by *Botrytis cinerea* and blue mold caused by *Penicillium expansum* on apple fruit, and the associated defense responses implicated were assessed (Abouraicha *et al.*, 2015, 2016). Ulvan (algae derived glyco product) activated a large set of related defense enzymes and accumulated a variety of resistance substances induced the resistance to anthracnose caused by *Colletotrichum lindemuthianum* in beans (*Phaseolus vulgaris*) (Paulert *et al.*, 2009; de Freitas and Stadnik, 2012), and protected from Fusarium wilt in seedlings of tomato (*Lycopersicon esculentum*) (El Modafar *et al.*, 2012). Pre-treatment of wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) plants with ulvan obtained from green macroalgae *Ulvan fasciata* significantly reduced the symptom severity of *Blumeria graminis* infection (Paulert *et al.*, 2010).

The application of algae derived agricultural biostimulants on crop plants produced numerous benefits in the midst of reported effects including enhanced seed germination, increased shooting proliferation, enhanced rooting (Hernandez-Herrera *et al.*, 2013; Satish *et al.*, 2015, 2016), higher crop and fruit yields, enhanced photosynthetic activity, salinity, drought and freezing tolerance, and resistance to various bacteria, fungi and viruses (Sharma *et al.*, 2014). We examined

the various marine macroalgae extracts in bioassays of *Solanum trilobatum* and *Eleusine coracana* seed germination, growth assays and *in vitro* regeneration including *Bacopa monnieri* (Satish *et al.*, 2015, 2016; Rency *et al.*, 2017). In the past, marine algae extracts have been successfully affianced as biostimulants in numerous plants, such as *Arabidopsis thaliana* (Khan *et al.*, 2011) and *Nicotiana tabacum* (Sanderson and Jameson, 1986) and *Glycine max* (Stirk and Van Staden, 1997) for the detection of cytokinin-like activity. Marine algae or their substances are applied successfully for the improvement of vegetables like *Brassica oleracea* (Abetz and Young, 1983), *Lactuca sativa* (Abetz and Young, 1983; Crouch *et al.*, 1990), *Beta vulgaris* (Featonby-Smith and Van Staden, 1983b), *Cucumis sativus* (Nelson and Van Staden, 1984; Jayaraman *et al.*, 2011), *Daucus carota* (Jayaraj *et al.*, 2008), *Lycopersicon esculentum* (Featonby-Smith and Van Staden, 1983a; Finnie and Van Staden, 1985; Kumari *et al.*, 2011; Zadope *et al.*, 2011; Basher *et al.*, 2012; Vinoth *et al.*, 2012, 2014; Hernandez-Herrera *et al.*, 2013; Briceno-Dominguez *et al.*, 2014), *Brassica rapa* (Chinese cabbage) (Sharma *et al.*, 2012), *Abelmoschus esculentus* (Papenfus *et al.*, 2013) and *Brassica napus* plants (Ferreira and Lourens, 2002). Other than this, pulses such as *Phaseolus vulgaris* (Featonby-Smith and Van Staden, 1984; Beckett *et al.*, 1994), *Phaseolus acutifolius* (Beckett *et al.*, 1994), *Vigna sinensis* (Sivasankari *et al.*, 2006), *Glycine max* (Rathore *et al.*, 2009) and *Vigna mungo* (Sharma *et al.*, 2012; Selvam and Sivakumar, 2013; Briceno-Dominguez *et al.*, 2014), in cereals like *Zea mays* (Jeannin, 1991), *Hordeum vulgare* (Steveni *et al.*, 1992) and *Triticum aestivum* (Beckett and Van Staden, 1989; Kumar and Sahoo, 2011), horticultural crops like *Fragaria ananassa* (Spinelli *et al.*, 2010) and in *Malus domestica* (Spinelli *et al.*, 2009) seaweed extracts applied successfully in different forms.

Marine algae extracts elicit a wide range of responses in the different plants

spp., through increased chlorophyll content, enhanced nutrient uptake, augmented flower and fruit set leading to elevated yields, delayed senescence and longer shelf life of fruits (Briceno-Dominguez *et al.*, 2014). In addition, the chemical breakdown of marine algae and their bioactive compounds has made known the standpoint of a wide range of substances such as gibberellins, auxins and cytokinins which are stimulating plant shoot and root growth, maturation and production (Hernandez-Herrera *et al.*, 2013; Satish *et al.*, 2015). The supplementation of algae based extracts showed positive response to the growth of various vegetables, fruits and other food crops since micro (Cu, Zn, B, Mn, Co and Mo), macro (Ca, K and P) nutrients, amino acids, organic acids, vitamins, antioxidants and complex minerals are available in their extracts (Hernandez-Herrera *et al.*, 2013; Briceno-Dominguez *et al.*, 2014; Satish *et al.*, 2015, 2016; Rency *et al.*, 2017). More or less, around 15 million metric tons of marine plant products are emerging every year (FAO, 2006), a substantial percentage of which distributed for nutrient supplementation and as biostimulants or biofertilizers in worldwide agriculture. At present, the advanced crop growing is paying attention for existing biotechnologies where it would consent for a concession in the application of chemicals without toxic effects on crop growth and yield as well as the farmers' earnings (Hernandez-Herrera *et al.*, 2013). These biostimulants can be useful as an alternative to herbs and pesticides, or used in conjunction through synthetic/artificial crop protection goods and as plant growth stimulants (Satish *et al.*, 2015, 2016), and they play a role in sustaining the crop production levels, high yield, health and quality (Sharma *et al.*, 2014), and marine algae or their derivatives are remaining largely unexploited universally.

7. Future prospects

Although a little information on the effects of glucuronan and its oligomer is already available on biological activities in plants (El Modafar *et al.*, 2012; Caillot *et al.*, 2012; reviewed above) there is a lack of studies regarding plant-pathogen models (Abouraicha *et al.*, 2017). Research outputs to decrease use of synthetic fertilizers and chemical pesticides for growing agricultural crops through the invention of novel natural compounds is immediately required in order to assemble the needs of sustainable agriculture and to counter to the increasing demand for pesticide-free food (Abouraicha *et al.*, 2017). Worldwide environmental demonstration in relation to the exhaustion of natural resources and industrialized pollution had led to the improvement of more renewable resources such as algal biomass, which is translated in elevated aquaculture assembly rates of 6.4 million tonnes in 2000 where it was 20.8 million tonnes fresh weight in 2012. Since the potential ecological and economic payment becomes evident, the research institutions, government and industries have been showing special interest towards developing the algal biomass (Kilinc *et al.*, 2013; Taelman *et al.*, 2015).

For the past few years, PBR systems have been improved through adopting photosynthesis in marine algae and it is a positive application of a photovoltaic PBR system mimicking a plant. The efficiency and miniaturization of algal PBRs are a different and novel challenge and the PBR culture systems are extraordinarily scaled up with respect to the open cultures.

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Biotransformation of Various Wastes into a Nutrient Rich Organic Biofertilizer - a Sustainable Approach towards Cleaner Environment

Geetha Karuppasamy¹, Michael Antony D'Couto^{1,2}, Sangeetha Baskaran^{1,3} and Anant Achary^{1,*}

¹*Department of Biotechnology, Centre for Research, Kamaraj College of Engineering and Technology, K.Vellakulam-625701, Near Virudhunagar, Madurai District, Tamil Nadu, India;* ²*JLL-Jones Lang LaSalle, TVH Belicia Towers, Thandavarayan Street, Mandavelipakkam, Raja Annamalai Puram, Chennai-600028, Tamil Nadu, India;* ³*Department of Biotechnology, St. Joseph's College Of Engineering, Old Mamallapuram Road, Semmencherry, Kamaraj Nagar, Semmancheri, Chennai-600119, Tamil Nadu, India;* * *Correspondence: achyanant@yahoo.com; Tel: 91-4549-278-171*

Abstract: Environmental degradation is one of the main threats confronting the world and the widespread use of chemical fertilizers contributes essentially to the degeneration of the environment through exhaustion of fossil fuels, generation of carbon dioxide (CO₂) and contamination of water bodies. Excessive use of fertilizers has adversely affected agricultural productivity causing soil degradation. Biotransformation through vermicomposting implies the production of nutrient-rich excreta of worms. After earthworms digest organic matter, they excrete a high-nutrient product known as Castings. Sustainable development can be achieved by providing adequate quantity of food for which agricultural land management is an important aspect. Earthworms are known to consume all types of organic wastes including vegetable waste, wastes generated by pulse and rice processing industries and other organic wastes. The food passes through the digestive tract and the worms secrete chemicals that break down organic matter into sustainable nutrition. Vermicompost is a peat like material consisting of excellent porosity, structure, aeration and moisture holding capacity that makes it a good organic manure for growing plants. Another important property of vermicompost is its vast surface area that provides strong absorbability and nutrient retention ability. Vermicompost increases soil fertility, enhance plant growth and suppress the population of plant pathogens and pests. As a soil conditioner, vermicompost is healthier to traditional compost for its capability to improve and enhance soil configuration and its water-holding capacity. Thus vermicomposting can be proposed as a cost effective and environment friendly method for efficient utilization of various organic wastes. This will promote healthy plant growth and aid in sustainable management of agricultural lands with cleaner environment.

Keywords: Biotransformation; earthworms; organic waste; vermicomposting; vermiwash

1. Introduction

With the increase in the population there has been a tremendous increase in generation of a variety of wastes. Although various measures are in place to handle the wastes generated by domestic

sector and industrial sector, the disposal of wastes is still a prime concern. There is an estimate that India produces approximately 3000 million tons of wastes per annum and among that more than 60% is found to be decomposable. Vegetable wastes are one of the major sources of

municipal wastes. The disposal of biodegradable solid wastes from domestic, agricultural and industrial sources has caused ever-increasing environmental and economic problems (Garg *et al.*, 2006).

Moreover, an additional key threat is the environmental degradation due to the extensive use of chemical fertilizers that has led to deterioration of the environment through generation of carbon dioxide (CO₂), exhaustion of fossil fuels, and water resources being contaminated. Agricultural productivity also has taken a heavy toll due to disproportionate use of fertilizers leading to soil degradation.

Recycling of wastes through biotransformation of various biodegradable wastes can reduce the problem of non-utilization of wastes. Locally available organic wastes of anthropogenic nature, domestic waste and agricultural lignocellulosic waste products can be used as biofertilizer as an alternative to chemical fertilizers. Vermicomposting employing earthworm as decomposers, for degradation and recycling, may be used to enhance the production of crops which are free from pollution and health hazard (Bakthvathsalam and Ramakrishnan, 2004). Preserving the quantity and quality of soil is one of the main objectives of current efforts to make agriculture more "sustainable".

2. Current waste disposal practices & its impact

Currently, waste is a major concern worldwide becoming exceedingly significant in developing countries like India, China as well as in Europe. Waste can be classified into industrial, agricultural, sanitary and solid urban residues on the basis of their origin. There might be a significant change in their distribution depending on the country. Waste generated by various food processing industries is a good example of globally generated waste on a large scale. Waste of this type has become a great source of concern as in certain cases it is found to be over 50%

of the total waste produced in countries. It has also been noted that 60% of food processing industry waste belongs to organic matter.

Global urbanization has led to increase in the volume of solid wastes. According to a survey conducted in 1990, about 1.3 billion metric tons of municipal solid waste was generated globally (Beede and Bloom, 1995). In today's scenario, the generation of solid waste per year equals to 1.6 billion metric tons approximately. A significant amount of capital is being invested into managing such huge volumes of solid waste suggesting that solid waste management (SWM) has become a large, complex and costly service.

The solid waste and its management at various stages are chiefly affected by the ever growing population. The numbers of households owing to the increase in population also has an important role in generation and collection of the solid waste. Solid waste is mainly collected by municipalities and the uncollected waste, approx. 31% to 49%, is left on street or road corners, open spaces like vacant plots that pollute the environment on continuous basis. The collected waste is usually disposed of within or outside the municipal limits into low lying areas like ponds etc, without any treatment except separation of recyclable waste by scavengers.

Extensive research around the world today is being carried out to find a solution for utilization of various agricultural residues as energy sources. A simple example is the sugarcane processing industries. Sugarcane bagasse and sugarcane agriculture residues are the two main residues of sugar and ethanol production process. Sugarcane bagasse is the fibrous waste that remains after recovery of sugar juice via crushing and extraction. It is one of the principal fuels used around the world in the sugarcane agro-industry because of its well-known energy properties. However, the bagasse management and disposal practices employed by the

sugar agro-industry have, in most cases, remained the same as those used back in the early 19th century leading to enormous amount of bagasse being disposed (Faria *et al.*, 2012). This bagasse can be an excellent material for biotransformation by vermicomposting. A similar scenario is also found in industries dealing with pulses and grains. The amount of waste generated as husk and bran is enormous. Although the husk and bran are being widely used in various industrial applications like industrial fuel, activated carbon, as pet food fiber, substrate for various fermentation processes etc, there is still lot of waste that is left unused.

Consumption of fruits and vegetables has radically increased in the various nations by more than 30% during the past few years. It is also projected that approximately 20% of all the fruits and vegetables produced is lost each year due to spoilage. Also, vegetable markets in various cities & towns are known to produce significant amount of non-edible vegetable wastes. As mentioned earlier, solid waste management of spoilt fruits and vegetables is one of the biggest problems faced today by all the cities including collection, transportation and disposal of the waste. These wastes are usually discarded in the market itself and allowed to rot. However, this discarding leads to production of hazardous ecological impacts (Kumari, 2013).

The poultry industry is one of the biggest and fastest growing livestock production systems in the world (Edwards and Daniel, 1992). As per the reports of Foreign Agricultural Service in 1992, internationally, approximately 40 million metric tons of poultry meat and 600 billion eggs were produced. Although reasonably flourishing, the poultry industry is at present facing a challenging environmental problem. From an agricultural standpoint, the role of poultry wastes in the contamination of soil and groundwater, the eutrophication of surface waters by nitrogen and phosphorus, and the fate

of pesticides, heavy metals, and pathogens polluting the soil via poultry wastes are the central environmental issues at the present time.

3. Role of chemical fertilizers in environmental degradation

3.1. Nutrient requirements for plant growth

For the survivability and growth of a plant, 16 essential nutrients are required including carbon, hydrogen, oxygen, nitrogen, phosphorous, potassium, magnesium, calcium and sulphur, iron, zinc, copper, manganese, boron, chlorine and molybdenum. Air, water and sunlight provide the necessary oxygen, carbon, hydrogen and energy.

3.2. Chemical fertilizers – a threat

Current practices mainly use the chemical fertilizer to accomplish the nutrients requirement of a growing plant which has a serious impact on the soil and water bodies. Excessive utilization of chemical fertilizer leads to increased salinity of soil which is one of the major threats causing lower productivity in the soil. Some of the serious impacts of using chemical fertilizers are:

- Pollution of ground & surface water
- Soil fertility is reduced leading to reduced food production
- Depletion in the soil microbial ecosystem
- Ground water pollution and destruction of the aquatic life
- Loss of terrestrial and aquatic biodiversity
- Depletion of the Ozone leading to global warming

Exhaustive cropping involving continuous use of high levels of chemical fertilizers (CF) often leads to nutritional disparity in soil and decline in crop productivity (Nambiar, 1994). Numerous properties characterizing the status of soil microbial biomass, activity and nutrient

content have been suggested as indicators of soil quality (Doran and Parkin, 1994). Although microbial biomass only forms a small fraction of soil organic matter, it contributes to agricultural sustainability because its high turnover rate is responsible for nutrient release and therefore promotes plant uptake (Smith *et al.*, 1993).

Conventional agro-ecosystems have been characterized by high input of chemical fertilizer instead of organic amendments, leading to deterioration of soil quality due to reductions in soil organic matter. With increasing global concerns about energy crisis and environmental protection, it is becoming more important to rely on locally abundant agricultural bioresources than on chemical fertilizers. Recent studies have focused on re-considering traditional fertilization practices to enhance soil organic input by amendment of organic fertilizers like vermicompost.

4. Biological decomposition of wastes

The breakdown of raw organic materials to a finished compost - a process known as decomposition - is a complex gradual process wherein both chemical and biological processes take place in order to mineralize the organic matters. Generally, biological degradation of organic material takes place through two distinct pathways:

i) Anaerobic digestion: Anaerobic digestion may be defined as the breakdown of organics in the absence of oxygen under controlled conditions. Here, fermentation of waste results in the formation of ammonia-like substances and hydrogen sulfide. Organic wastes with high degradability are easily degraded through anaerobic digestion. Bacterial species capable of degrading targeted organics are involved in this process along with thorough mixing for efficient substrate conversion (Figure 1). The carbon content released as biogas containing methane, carbon dioxide and energy is represented using the equation:

$$C_6H_{12}O_6 \rightarrow 3CO_2 + 2CH_4 + 393kJ \quad \dots 1$$

The drawback of this technique is that it cannot be used for mixed domestic waste composting.

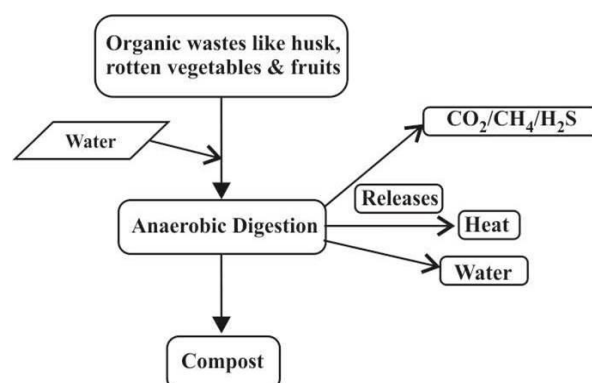


Figure 1: Schematic representation of anaerobic digestion process for composting of agricultural wastes.

ii) Aerobic digestion: Aerobic digestion may be defined as a biological degradation process where vigorous humification and pasteurization of organic residues takes place. The process requires air breathing microbes like bacteria, fungi, actinomycetes, mesophilic-exothermic microbes and thermophilic microorganisms that flourish in elevated temperature of greater than 60°C. Mineralization of biodegradable organic matter takes place leading to release of carbon dioxide, water and energy (Figure 2). Conversion of

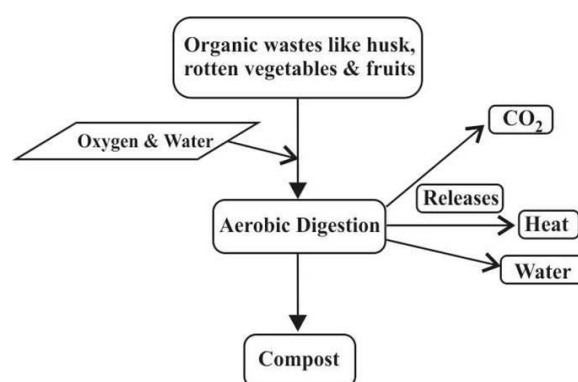
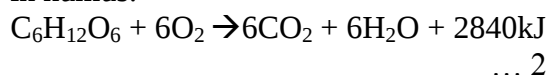


Figure 2: Schematic representation of aerobic digestion process for composting of agricultural wastes.

residual organic components to humic acids results in stabilization of the process. As the end result, the heterogeneous waste is transformed into a homogeneous

and valuable organic fertilizer that is rich in humus.



Compared to chemical fertilizers, compost is an excellent product that retains most of the original nutrients beneficial for the increase of soil's organic and nutrient constituents. Application of compost can improve structure and fertility of the soil. There are basically four important parameters that must be considered for the evaluation of quality of compost and process performance. They are: volatile solids, respiration rate, germination tests and pathogen indicators.

5. Vermicomposting

Vermicomposting is an ecological stabilization process involving the breakdown of organic waste by the joint action of earthworms and mesophilic microorganisms. Earthworms require an environment conducive for microbial degradation and maintenance of biochemical processes for enhanced microbial decomposition. Various intestinal microflora of earthworms are transferred to the compost matrix along with their gut enzymes that play an important role (Whiston and Seal, 1988). Furthermore, earthworms are also known to augment the microbial activities by improvement of the environment necessary for their growth (Syers *et al.*, 1979; Mulongoy and Bedoret, 1989). Through the process of vermicomposting, wastes are converted to a better homogenized, nutrient rich and well stabilized product. Several studies reveal that vermicomposting can be used as an effective technique for the treatment of wastes rich in pathogens (Eastman *et al.*, 2001). Vermicomposting gives a better end product than composting due to the enzymatic and microbial activity that takes place during the process (Bajsa *et al.*, 2003). Various studies indicate that vermicomposting can attain harmless pathogen levels which may be aided by the microbial and enzymatic activity. An added advantage of this pro-

cess is the conversion of important plant nutrients into a more soluble state (Nair *et al.*, 2006).

Several parameters can be used for the evaluation of the vermicomposting process, like, survival of the worms, biomass growth, and increase in worm population. Since earthworms are known to feed on the pathogens present in the waste used, vermicomposting process guarantees pathogen removal. Hence, as per USEPA, Vermicomposting has been recognized as 'Class A' stabilization process (Eastman *et al.*, 2001). Another advantage of biotransformation of waste through vermicomposting is the loss of moisture to yield a drier product. High concentration of moisture in the compost can lead to process failure. In vermicomposting, the worm burrows act as channels for air passage, hence, this can support higher humidity.

The main problems encountered with composting of many organic wastes are their high moisture content, need of bulking substrate and components undesirable for worm consumption. As a result, composting of raw organic wastes requires constant monitoring of moisture level, composition of the waste, C/N ratio and the composting period. This has been overcome by the process of pre-digestion of organic waste before it can be utilized for vermicomposting. Geetha *et al.*, (2016) and Nair *et al.*, (2006) have carried out pre-digestion of the non-edible vegetables waste and kitchen wastes respectively, in order to achieve a better quality of vermicompost. Pre-digestion prior to vermicomposting was helpful in pH, moisture and waste stabilization. Pre-digestion was also found to effectively reduce the pathogen load from the vermicompost.

5.1. Earthworms

Earthworms belong to kingdom Animalia, phylum Annelida, class Clitellata and subclass Oligochaetae. They are known as soil forming organisms inhabiting almost all part of the earth. They can

be found in different groups of varying size, shape, colouration, life span, feeding habit and depth in the soil where they are found. The shape of the worm is cylindrical with segmented body tapering off at both ends. The segments are separated by fluid-filled compartment that surrounds a central digestive tract.

More than 1800 known species of earthworm have been found all over the world (Minnich, 1977) and they can be subdivided in three basic groups: *Epigeics*, *Endogeics* and *Anecics*. Although there are probably many species of earthworms available, only a few have been utilized for biotransformation of organic waste processing. The most commonly used species of earthworms include *Eisenia foetida* (Red wiggler), *Lumbricus rubellus* (Red worm), *Eisenia Andrei* (Red tiger), *Perionyx excavates* (Blue worm), *Eudrilus eugeniae* (African night crawler), *Enchytraeids* (White worm), *Dendroba enaveneta* and *Perionyx hawayana*. Although all the above mentioned species may be used for vermicomposting, *Eisenia foetida* and *Lumbricus rubellus* are the most commonly used as it is easy to replicate the suitable environmental condition for their growth and regeneration. Among the two species, *Eisenia foetida* has been proved best for vermicomposting of any organic waste (Edwards and Bater, 1992) because the growth and reproduction of these worms is quite rapid. *Eisenia foetida*, commonly known as Red wiggler, is an epigenic worm that favours living in organic manure or compost. It has the ability to process large quantities of organic matter as under ideal conditions; it is known to consume food as much as its body weight each day.

5.2. Vermicomposting process

The process of vermicomposting can be carried out either in batch or in continuous modes. The time taken for the completion of decomposition process may vary depending on the time taken by the earthworms to ingest the feed, digest and

excrete. The following steps are involved in vermicomposting process (Abbasi et al., 2008):

- i. Ingestion and breaking down of the ingested organic waste by the action of earthworm's gizzard, located next to the mouth of the worm.
- ii. Digestion of the broken down particles by the action of enzymes and microbes while it passes through the earthworm's body.
- iii. Exit of the digested matter as "Vermicast" after few hours of ingestion.

Vermicomposting bins (Figure 3) are set up using Vermitech pattern (Geetha et al., 2016). The plastic or ceme-

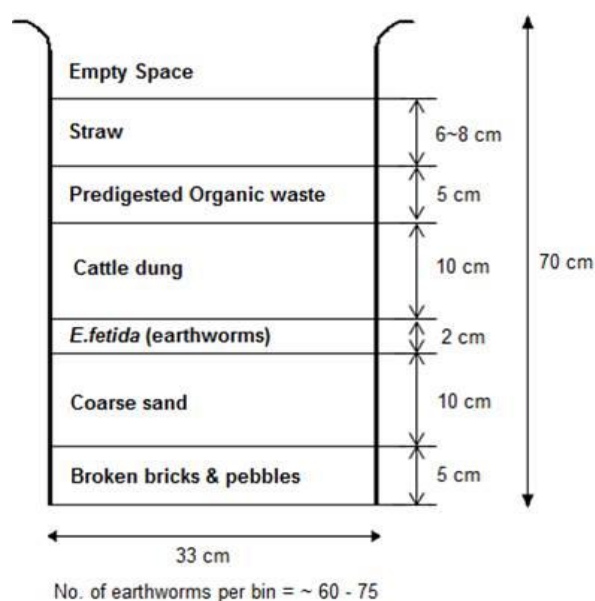


Figure 3: Schematic representation of design of vermicompost bin (Geetha et al., 2016).

-nt bins are placed in a shaded elevated area on a pedestal of bricks for effective water drainage. The basal layer of the vermibed comprises of broken bricks and stones above which a layer of sand up to the height of 10 cm is set up to ensure proper filtration and drainage. This is followed by first a 10 cm high layer of cow dung (3kg) and 5cm high layer of pre-digested organic waste. A 6~8 cm layer of straw is added to cover the bedding material that helps in retaining moisture. Ap-

proximately 60-75 earthworms are inoculated in the composting bins. The vermicomposting units are kept in shade and covered with a mesh. Water is sprinkled to maintain sufficient moisture. For operative collection of wormy wash, a small hole is drilled near the base of composting bin and a tube is attached to it. Turning of the vermicomposting pile must be carried out periodically to ensure proper aeration.

5.3. Advantages of waste management by vermicomposting

Composting is known as an effective biotransformation technique for treating organic wastes, following nature's way of recycling. Vermicomposting requires very less resources like water, energy and land/space required for treatment of per unit of bio-waste as compared to aerobic composting. It is a rapid, cost effective and sustainable alternative for transformation of organic waste, carried out by earthworms, leading to formation of a product rich in plant nutrients and humic acids. The compost thus generated has the ability to hold nutrients for a longer period without unfavorably impacting the environment. No curing is necessary for vermicompost as it is highly rich in beneficial microorganisms. The overall time required for processing of waste is therefore reduced to a great extent, resulting in non-toxic by-products. Gardeners, all over the world, highly prefer the application of vermicompost over chemical fertilizers; hence, vermicompost is finding a significant market value (Sudhakar *et al.*, 2002).

Application of vermicompost has been proved to improve the texture, structure, aeration, fertility and water holding capacity of soil. This way most of the valuable nutrients that are taken out of the soil during crop cultivation are replenished. Also, vermicompost addition is known to enhance plant-root developments that help in control of soil erosion. Vermicompost are also known to be rich

in various enzymes, high quality organics (humus) and plant growth regulators.

6. Application of biotransformed waste for better agricultural productivity

One of the biggest environmental challenges that the world is facing today is solid waste management. A sustainable approach towards management of this problem may be the use of biotransformation technique to treat and convert organic waste into vermicompost. Composting is the most cost-effective and ecological option for management of various organic wastes since it is easily operable and can be carried out in a small scale level with proper management of the process to obtain a good quality product. A list of various wastes converted to vermicompost is given in Table 1. Although, husk is abundantly available as lignocellulosic waste, the problem with using husks as vermicomposting feedstock is its high initial carbon (lignin and cellulose) contents, which impedes the composting process (Kumar *et al.*, 2013). Hence, the substrate requires amendments to achieve an optimum range of C/N ratio to attain optimal process efficiency (Goyal *et al.*, 2005). This can be achieved with the help of pre-digestion of husks along with other organic wastes (such as non-edible vegetable waste) as amendment, prior to vermicomposting. This will help in conversion of husk into biofertilizer within few months.

Integrated use of fly ash along with organic wastes has been effectively used in increasing the yield of crops when compared to continuous use of chemical fertilizers alone (Rautaray *et al.*, 2003). This may be due to beneficial effects on rice and residual effects on mustard. According to Rautaray *et al.* (2003) greater crop yield was related to higher uptake of nutrient. Further to the yield advantage, a better soil chemical properties were also noted namely pH, organic carbon and available N, P and K as compared to the soils where chemical fertilizers were con-

tinuously used. When fly ash was used in combination with organic waste during vermicomposting, the end product obtained gave better plant growth than with chemical fertilizers alone.

Vigna radiata (Green gram) when grown in soil amended with a combination of vermicompost and wormy wash were found to develop nodes and new leaves at a significantly higher rate when compared to control (Geetha et al., 2016). This might be due to increase in bioavail-

ability of macro and micronutrients from vermicompost and wormy wash (Erich et al., 2002). Vermicompost and wormy wash augments crop growth and yield when added to soil (Lalitha et al., 2000). According to the studies conducted by Atiyeh et al. (2001), and Suthar (2009), addition of vermicompost in bedding media enhanced seed germination and growth leading to overall increase in plant productivity.

Table 1: List of organic wastes converted to vermicompost

No.	Organic waste	Earthworm employed	Outcome	Reference
1.	Non-edible vegetables from market	<i>Eisenia foetida</i>	Enhanced growth of <i>Vigna radiata</i> (green gram)	Geetha et al., 2016
2.	Kitchen waste	<i>Eisenia fetida</i> & <i>Lumbricus rubellus</i>	Effect of thermocomposting prior to vermicomposting	Nair et al., 2006
3.	Industrial sludge	<i>Eisenia foetida</i>	Removal of the heavy metals from electronic industrial sludge	Shaymaa et al., 2010
4.	Vegetable wastes	<i>Eisenia foetida</i>	Composting with minimum resources leading to zero waste generation	Sibi and Manpreet, 2011
5.	Farm garbage	<i>Eisenia fetida</i> , <i>Eudrilus eugeniae</i> & <i>Peronyx excavates</i>	Odourless product with high nutrient status.	Indrajeet et al., 2010
6.	Agriculture waste from market yard	<i>Eisenia Foetida</i> & <i>Eudrilus eugeniae</i>	Simplest, scientific, economic and environmental friendly way to transform waste materials into compost through vermicomposting by using an exotic species of earthworm	Mane and Raskar, 2012
7.	Fruit waste	<i>Eisenia Foetida</i> & <i>Eudrilus eugeniae</i>	Degradation strategy of organic waste	Seetha et al., 2012
8.	Coconut waste	<i>Eudrilus eugeniae</i>	Efficient method to convert coconut waste into valuable by-product	Tahir and Hamid, 2012
9.	Rice husk	<i>Eudrilus eugeniae</i>	Bio-transforming RH into value-added material,	Lim et al., 2012
10.	Water hyacinth and Grass clippings	<i>Eisenia fetida</i>	Nutrient rich vermicompost	Ansari and Rajpersaud, 2012

7. Concluding remarks

Environmental degradation is a major cause of concern challenging the world. Owing to improper waste management amenities and treatment, discarding of organic wastes from various sectors like domestic, agricultural and industrial sources has become a source of distress causing serious environmental complications. As a result of fast growing population, there is also significant increase in the generation of various wastes all over the world. Vermicomposting, being an environmental-friendly technique, is an attractive method for conversion of waste into wealth. In today's world of organic products, people are becoming increasingly aware of the importance of converting our waste into valuable product that will not only solve the problem of waste disposal but also help the agriculturalists in acquiring a safe and cost-effective crop promotion technique.

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Bacterial Endophytes as Biofertilizers and Biocontrol Agents for Sustainable Agriculture

Amrutha V. Audipudi¹*, Bhaskar V. Chakicherla² and Shubhash Janardhan Bhore³

¹Department of Microbiology, Acharya Nagarjuna University, Nagarjuna Nagar 522510, Andhra Pradesh, India; ²Department of Botany, V.R College, Affiliated to V.S. University, Nellore 524002, A.P, India; ³Department of Biotechnology, Faculty of Applied Sciences, AIMST University, Bedong-Semeling Road, 08100 Bedong, Kedah Darul Aman, Malaysia;

*Correspondence: audipudiamrita@gmail.com; Tel.: +91 9440995842

Abstract: Plant health and development promoted through microbial interactions have been the main motif for sustainable agriculture. To find new and beneficial endophytic microorganisms from plants of different ecosystems is highly considerable because endophytic bacteria are not restricted to a specific species but showed a wide range of host diversity. Endophytes colonize an ecological niche similar to that of phytopathogens and baffle disease development through endophyte-mediated de novo synthesis of novel compounds and anti-fungal metabolites. Seedling emergence, plant growth and plant's establishment under adverse conditions can be accelerated by endophytes. Endophytic *Pseudomonas sp.* and *Bacillus sp.* recorded a significant improvement in morphological characters and ISR in the plantlets. Endophyte fortifies plant cell wall strength, alters host physiology and metabolic responses thereby enhance the defense mechanism by the synthesis of different metabolites such as phenolic compounds, pathogenicity related protein (PR-1, PR-2, PR-5), oxidative stress enzymes (chitinases, peroxidases, polyphenyloxidase, phenyl alanine ammonia lyase, Oxidase and/or chalcone synthase, phytoalexins etc. The contribution of endophytes as biological fertilizers is highly significant because of their metabolic acclimatization in the host plant with mutualism. Endophytic microbes must be properly selected, combined and formulated with respect to the environmental conditions for development of efficient endophytic biofertilizers that can very much contribute to the enhanced food production in the world. This review aims to provide an overview on bacterial endophytes and their potential application for sustainable agriculture.

Keywords: Agriculture; biocontrol; endophytes; endophytic bacteria; induced systemic resistance; phytopathogens; plant growth promotion; sustainable development

1. Introduction

Agriculture and agri-food sector is expected to move towards environmentally sustainable development by increasing the productivity and protecting the natural resource base for future generations. Greater productivity and competitiveness are anticipated to come from increased efficiency through the acquisition and management of new biotechnologies and crop production strategies (Stutz *et al.*,

1999). Plant-microbe interactions that promote plant health and development have been the subject of considerable study for sustainable agriculture. A renewed interest in the internal colonization of healthy plants by non-rhizobium bacteria and exploitation of their potential in agriculture becomes apparent (Fahey *et al.*, 1991; Kloepper *et al.*, 1992; Turner *et al.*, 1993). Rhizosphere bacteria which can easily colonize the internal roots and stems are major source of endophytes

(Figure 1) and often phyllosphere bacteria may also be a source of endophytes (Hallmann *et al.*, 1997; Germaine *et al.*, 2004). Though each individual plant exists on the earth is a host to one or more endophytes (Strobel *et al.*, 2004), only a few of these plants have ever been completely studied to their endophytic biology and to find novel beneficial endophytic microorganisms.

2. Endophytic bacterial diversity in the host plants

Endophytic bacteria were isolated from both monocotyledonous and dicotyledonous plants (Table 1) ranging from woody trees to herbaceous crops such as prairie plants, agronomic crops, tuberous crops and grasses (McInroy and Kloepper, 1995; Gutiérrez-Zamora and Martínez-Romero, 2001; Germida and

Siciliano, 2001; Zinniel *et al.*, 2002; Sessitsch *et al.*, 2002; Dent *et al.*, 2004; Sun *et al.*, 2008). More than 200 bacterial genera from 16 phyla have been reported as endophytes since the first report of endophytic bacteria (Samish *et al.*, 1963a) and include both culturable and unculturable bacteria (Sun *et al.*, 2008; Berg and Hallmann, 2006; Mengoni *et al.*, 2009; Manter *et al.*, 2010; Sessitsch *et al.*, 2012). Most predominantly studied endophytes belong to three major phyla (*Actinobacteria*, *Proteobacteria* and *Firmicutes*) and include members of *Streptomyces* (Suzuki *et al.*, 2005), *Azoarcus* (Krause *et al.*, 2006), *Acetobacter* (renamed as *Gluconobacter*) (Bertalan *et al.*, 2009), *Pseudomonas*, *Serratia*, *Stenotrophomonas* (Ryan *et al.*, 2009), *Enterobacter* (Pedrosa *et al.*, 2011). Bacteria which are ubiquitous in the soil/ rhizosphere represent the main source of endophytic

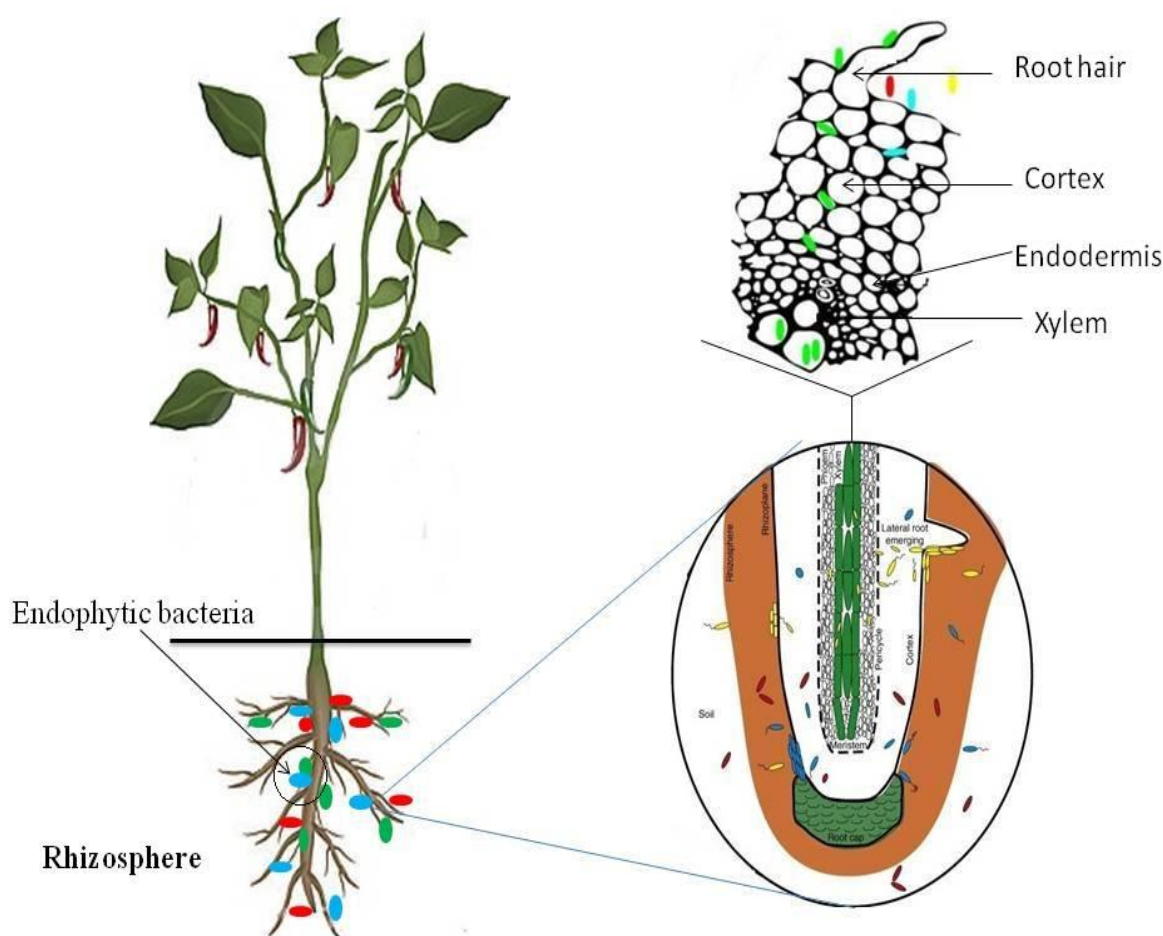


Figure 1: Plant colonization routes by endophytic bacteria.

Table 1: Monocotyledonous and dicotyledonous plants that harbor bacterial endophytes (Sudhir and Audipudi, 2014)

Plant species	Endophytes	Reference
Rice (<i>Oryza sativa</i> L.)	<i>Rhizobium leguminosarum</i> , <i>Pseudomonas</i> <i>Azorhizobium caulinodans</i> , <i>Sphingomonas paucimobilis</i> <i>Chromobacterium violaceum</i> , <i>Sphingobacterium</i> sp. <i>Bradyrhizobium japonicum</i> <i>Serratia marcescens</i> <i>Serratia</i> sp. <i>Agrobacterium</i> , <i>Azorhizobium</i> , <i>Azospirillum</i> , <i>Bacillus</i> ,	Yanni et al., 1997, Engelhard et al., 2000, Phillips et al., 2000, Chantreuil et al., 2000 Gyaneshwar et al., 2001 Sandhiya et al., 2005 Reddy et al., 1997; Stoltzfus et al., 1997
Potato (<i>Solanum tuberosum</i> L.) tuber	<i>Actinomyces</i> , <i>Agrobacterium</i> , <i>Alcaligenes</i> , <i>Arthrobacter</i> , <i>Bacillus</i> , <i>Capnocytophaga</i> , <i>Cellulomonas</i> , <i>Clavibacter</i> , <i>Comamonas</i> , <i>Corynebacterium</i> , <i>Curtobacterium</i> , <i>Deleya</i> , <i>Enterobacter</i> , <i>Erwinia</i> , <i>Flavobacterium</i> , <i>Kingella</i> , <i>Klebsiella</i> , <i>Leuconostoc</i> , <i>Micrococcus</i> , <i>Pantoea</i> , <i>Pasteurella</i> , <i>Photobacterium</i> , <i>Pseudomonas</i> , <i>Psychrobacter</i> , <i>Serratia</i> , <i>Shewanella</i> , <i>Sphingomonas</i> , <i>Vibrio</i> , <i>Xanthomonas</i> , <i>Sinorhizobium meliloti</i> , <i>Paenibacillus odorifer</i> , <i>Enterobacter asburiae</i>	Hollis, 1951; de Boer I Copeman, 1974; Sturz, 1995; Sturz & Matheson, 1996; Sturz et al., 1998 Reiter et al., 2003 Asis and Adachi, 2003
Red clover (<i>Trifolium pratense</i> L.)	<i>Acidovorax</i> , <i>Agrobacterium</i> , <i>Arthrobacter</i> , <i>Bacillus</i> , <i>Bordetella</i> , <i>Cellulomonas</i> , <i>Comamonas</i> , <i>Curtobacterium</i> , <i>Deleya</i> , <i>Enterobacter</i> , <i>Escherichia</i> , <i>Klebsiella</i> , <i>Methylobacterium</i> , <i>Micrococcus</i> , <i>Pantoea</i> , <i>Pasteurella</i> , <i>Phyllobacterium</i> , <i>Pseudomonas</i> , <i>Psychrobacter</i> , <i>Rhizobium</i> , <i>Serratia</i> , <i>Sphingomonas</i> , <i>Variovorax</i> , <i>Xanthomonas</i>	Sturz et al., 1997 Sturz et al., 1998
Rough lemon (<i>Citrus jambhiri</i> Lush.)	<i>Achromobacter</i> , <i>Alcaligenes</i> <i>Moraxella</i> , <i>Acinetobacter</i> , <i>Actinomyces</i> , <i>Arthrobacter</i> , <i>Bacillus</i> , <i>Citrobacter</i> , <i>Corynebacterium</i> , <i>Enterobacter</i> , <i>Flavobacterium</i> , <i>Klebsiella</i> , <i>Providencia</i> , <i>Pseudomonas</i> , <i>Serratia</i> , <i>Vibrio</i> , <i>Yersinia</i> , <i>Rickeltsia</i> -like	Feldman et al., 1977; Gardner et al., 1982

Table 1: Continued...

Grapevine (<i>Vitis spp.</i>)	<i>Bacillus</i> , <i>Clavibacter</i> , <i>Comamonas</i> , <i>Curtobacterium</i> , <i>Enterobacter</i> , <i>Klebsiella</i> , <i>Moraxella</i> , <i>Pantoea</i> , <i>Pseudomonas</i> , <i>Rahnella</i> , <i>Rhodococcus</i> , <i>Staphylococcus</i> , <i>Xanthomonas</i>	Bell et al., 1995a; 1995b
Soybean	<i>Enterobacter sakazakii</i> <i>Enterobacter agglomerans</i> <i>Erwinia</i> sp. <i>Klebsiella oxytoca</i> <i>Klebsiella pneumoniae</i> <i>Pseudomonas citronellolis</i>	Kuklinsky-Sobral et al., 2004
Maize	<i>Burkholderia pickettii</i> <i>Enterobacter</i> spp. <i>Arthrobacter globiformis</i> <i>Microbacterium testaceum</i>	McInroy and Kloepper, 1995 Chelius and Triplett, 2000a Zinniel et al., 2002
Citrus plants	<i>Bacillus</i> spp. <i>Curtobacterium flaccumfaciens</i> <i>Nocardia</i> sp. <i>Methylobacterium mesophilicum</i>	Araujo et al., 2001, 2002
Carrot	<i>Pseudomonas putida</i> <i>Pseudomonas fluorescens</i> <i>Staphylococcus saprophyticus</i> <i>Klebsiella terrigena</i>	Surette et al., 2003
Sugar cane (<i>Saccharum officinarum</i> L.)	<i>Herbaspirillum rubrisulbalbicans</i> , <i>Acetobacter</i> , <i>Herbaspirillum</i>	Olivares et al., 1996 Cavalcante & Döbereiner, 1988; Gillis et al., 1989; Boddey et al., 1991; Dong et al., 1994; Ohvares et al., 1997
Corn (<i>Zea mays</i> L.)	<i>Bacillus</i> , <i>Burkholderia</i> , <i>Corynebacterium</i> , <i>Enterobacter</i> , <i>Klebsiella</i> , <i>Pseudomonas</i>	Lalande et al., 1989; Fisher et al., 1992; McInroy & Kloepper, 1995; Palus et al., 1996
Wheat	<i>Streptomyces</i>	Coombs and Franco, 2003a
Alfalfa (<i>Medicago sativa</i> L.)	<i>Erwinia-like</i> , <i>Pseudomonas</i>	Gagne et al., 1987
Coffee (<i>Coffea arabica</i> L.); Cameroon grass (<i>Pennisetum purpureum</i> Schumach)	<i>Acetobacter</i>	Jimenez-Salgado et al., 1997; Reis et al., 1994
Cotton (<i>Gossypium hirsutum</i> L.)	<i>Agrobacterium</i> , <i>Bacillus</i> , <i>Burkholderia</i> , <i>Clavibacter</i> <i>Erwinia</i> , <i>Serratia</i> , <i>Xanthomonas</i>	Misaghi & Donndelinger, 1990; McInroy & Kloepper, 1995
Cucumber (<i>Cucumis sativus</i> L.)	<i>Agrobacterium</i> , <i>Arthrobacter</i> , <i>Bacillus</i> , <i>Burkholderia</i> ,	McInroy & Kloepper, 1995

Table 1: Continued...

	<i>Chryseobacterium</i> , <i>Enterobacter</i> , <i>Pseudomonas</i> , <i>Stenotrophomonas</i>	
Hybrid spruce (<i>Picea glauca</i> x <i>Engelmannii</i>)	<i>Bacillus</i> , <i>Pseudomonas</i> , <i>Phyllobacterium</i> , <i>actinomyces</i> , <i>Staphylococcus</i>	O'Neill et al., 1992; Chanway et al., 1994
Kallar grass (<i>Leptochloa fusca</i> [L.] Kunth) root	<i>Azoarcus</i>	Reinhold et al., 1986; Reinhold-Hurek et al., 1993
Lodgepole pine (<i>Pinus contorta</i> Dougl. Ex Loud) root	<i>Bacillus</i>	Shishido et al., 1995
<i>Sorghum bicolor</i> L. Moench shoot	<i>Herbaspirillum</i>	James et al., 1997
Sugar beet (<i>Beta vulgaris</i> L.)	<i>Bacillus</i> , <i>Corynebacterium</i> , <i>Erwinia</i> , <i>Lactobacillus</i> ; <i>Pseudomonas</i> , <i>Xanthomonas</i>	Jacobs et al., 1985
Teosinte (<i>Zea luxurians</i> Itins and Doebley) stem	<i>Klebsiella</i>	Palus et al., 1996
Banana	<i>Azospirillum brasilense</i>	Weber et al., 1999
Marigold	<i>Citrobacter</i> sp. <i>Kocuria varians</i> <i>Microbacterium esteraromaticum</i>	Martínez et al., 2003 Sturz and Kimpinski, 2004
Banana, pineapple	<i>Azospirillum amazonense</i>	Weber et al., 1999
Sugarcane, coffee	<i>Gluconacetobacter diazotrophicus</i>	Cavalcante and Döbereiner, 1988; Jiménez-Salgado et al., 1997
Scots pine, citrus plants	<i>Methylobacterium extorquens</i>	Araujo et al., 2002; Pirttilä et al., 2004
Carrot, rice	<i>Rhizobium</i> (<i>Agrobacterium</i>) <i>radiobacter</i>	Surette et al., 2003
Kallar grass, rice	<i>Azoarcus</i> sp.	Reinhold-Hurek et al., 1993, Engelhard et al., 2000;
Yellow lupine, citrus plants	<i>Burkholderia cepacia</i>	Araujo et al., 2001; Barac et al., 2004
Banana, pineapple, rice	<i>Burkholderia</i> sp.	Araujo et al., 2001; Barac et al., 2004
Sugarcane, rice, maize, sorghum, banana	<i>Herbaspirillum seropedicae</i>	Olivares et al., 1996; Weber et al., 1999
Banana, rice, maize, sugarcane	<i>Klebsiella variicola</i>	Rosenblueth et al., 2004.
Citrus plants, sweet potato	<i>Pantoea agglomerans</i>	Araujo et al., 2001, 2002; Asis and Adachi 2003
Rice, soybean	<i>Pantoea</i> sp.	Kuklinsky-Sobral et al., 2004; Verma et al., 2004

Table 1: Continued...

Marigold (<i>Tagetes</i> spp.), carrot	<i>Pseudomonas chlororaphis</i>	Sturz and Kimpinski, 2004; Surette et al., 2003
Alfalfa, carrot, radish, tomato	<i>Salmonella enterica</i>	Cooley et al., 2003; Guo et al., 2002; Islam et al., 2004
Dune grasses (<i>Ammophila</i> <i>arenaria</i> and <i>Elymus mollis</i>)	<i>Stenotrophomonas</i>	Dalton et al., 2004
Maize, carrot, citrus plants	<i>Bacillus megaterium</i>	Araujo et al., 2001; McInroy and Kloepper, 1995; Surette et al., 2003
Grass <i>Miscanthus sinen-</i> <i>sis</i>	<i>Clostridium</i>	Miyamoto et al., 2004
Wheat, Scots pine	<i>Mycobacterium</i> sp.	Conn and Franco 2004; Prit- tilä et al., 2005
Citrus plants, maize	<i>Enterobacter cloacae</i>	Araujo et al. 2002; Hinton et al., 1995
Lettuce	<i>Escherichia coli</i>	Ingham et al., 2005
Wheat, sweet potato, rice	<i>Klebsiella</i> sp.	Engelhard et al., 2000; Iniguez et al., 2004;

colonizers (Hallmann and Berg, 2006). Earlier studies reported that endophytic population of *Bacillus polymyxa* inside the root is highly specific and less diverse than the root surface population, though there are different populations of *Bacillus polymyxa* in soil, rhizosphere, and rhizoplane of wheat field. It clearly indicates that endophytes appear to be originated from rhizosphere or rhizoplane (Mavingui et al., 1992; Germida et al., 1998).

Endophytic genera isolated from the interior of ginseng roots cultivated in three different areas showed marked differences in microbial community (Cho et al. 2007). Ryan et al., (2008) reported that endophytic bacteria in a single plant host not restricted to a single species but comprise several genera and species. Endophytic bacteria belong to one genera was not only restricted a specific host but also showed wide range of host diversity. Bacteria belonging to the genera *Bacillus* and *Pseudomonas* were identified as predominantly occurring endophytes (Seghers et al., 2004).

Jha and Kumar (2007) isolated and characterized 10 endophytic diazotrophic bacteria from surface-sterilized roots and culm of *Typha australis*. Long

et al., (2008) isolated 77 endophytic bacteria from roots, stems and leaves of *Solanum nigrum* grown in two different native habitats of Jena and Germany. Aravind et al., (2009) isolated 80 endophytic bacteria from different varieties of *Piper nigrum* L. cultivated in different regions of India. Bhore and Sathisha (2010) isolated 115 putative cultivable endophytic bacteria from leaves of 72 different plant species collected from northern part of Peninsular Malaysia. Magnani et al., (2010) isolated 32 endophytic bacteria from Brazilian sugarcane. A total of 264 colonies of endophytic bacteria were reported from leaves and roots of young radish (Seo et al., 2010).

Pereira et al., (2011) investigated endophytic bacterial diversity associated with the roots of maize through culture-dependent and culture-independent methods. *Enterobacter*, *Erwinia*, *Klebsiella*, *Pseudomonas*, and *Stenotrophomonas* genera belong to γ -Proteobacteria were reported as predominant group. Based on culturable component of the bacterial community genus *Bacillus* belong to Firmicutes was identified as another predominant group, while *Achromobacter*, *Lysinibacillus*, and *Paenibacillus* genera

were rarely found in association with the roots.

Yang *et al.* (2011) isolated 45 strains from stems and 27 strains from leaves of tomato as endophytic bacteria and reported that endophytic efficiency of bacteria in stem was higher than leaves. Out of 72 endophytic bacteria isolated from tomato one of the W4 was identified as *Brevibacillus brevis* W4 on the bases of 16S rDNA gene analysis and Biolog systemic analysis. Patel *et al.* (2012) isolated and characterized bacterial endophytes from root and stem of *Lycopersicon esculentum*. Out of 18 endophytic bacteria selected from tomato only one isolate HR7 was identified as *Pseudomonas aeruginosa* by 16S rDNA analysis.

Fisher *et al.* (1992) found that endophytic bacteria appear to be preferentially located in the lower part of the stems of corn, with a declining gradient running from the base to the top of the plant. Roots and other below ground tissues tend to yield the highest numbers of CFU of bacteria compared with above-ground tissues is an indicative of the upward path of bacteria (Table 2) from the roots into the stem during plant development (McInroy and Kloepper, 1994; Sturz *et al.*, 1997a; Rosenblueth and Martínez-Romero, 2004; Gagné *et al.* (1987).

Internal colonization of plant tissues by bacteria is primarily intercellular and xylem vessels act as reservoirs of internal populations of bacteria (Gardner *et al.*, 1982; Jacobs *et al.*, 1985; Sumner, 1990; Frommel *et al.*, 1991; Kloepper *et al.*, 1992; Bell *et al.*, 1995; Sprent and James 1995; Reinhold-Hurek and Hurek 1998a). Intracellular endophytic bacteria have also been found in the cytoplasm and vacuoles of epidermal cells (Quadt - Hallman and Kloepper, 1996), root hairs (Vance, 1983), and parenchyma cells (Jacobs *et al.*, 1985).

3. Endophytes in agriculture

The endophyte-plant interaction is one of the least studied biochemical systems in nature. Plants host to one or more endophytic microorganisms include fungi, bacteria and actinomycetes. Endophytes reside in the tissues beneath the epidermal cell layers (Stone *et al.*, 2000) are transiently symptomless and inconspicuous with several beneficial effects on plants (Hallmann *et al.*, 1997). More than thousand endophytic bacteria were reported in the last decade (Table 3). Like plant growth promoting Rhizobacteria (PGPR),

Table 2: Population densities of endophytic bacteria in host tissues

Part of the plant	Colony forming Unit (CFU)	Reference
Alfalfa xylem tissue	6.0×10^3 to 4.3×10^4 per g	Gagné <i>et al.</i> , 1987
Cotton xylem tissue	1×10^2 to 11×10^3 per g	Misaghi and Donndelinger, 1990
Sugar beet tissue	3.3×10^3 to 7.0×10^5 per g	Jacobs <i>et al.</i> , 1985
Potato tubers	0 to 1.6×10^4 per g	De Boer and Copeman, 1974

Table 3: Endophytic strains reported in between 2001-2007 (Sudhir, 2014)

Source	No of endophytes	Reference
Indian sugarcane	81 endophytic bacterial strains	Suman <i>et al.</i> , 2001
agronomic crop species	853 endophytic bacteria	Lodewyckx <i>et al.</i> , 2002
prairie plant species	27 endophytes	Zinniel <i>et al.</i> , 2002
<i>Daucus carota</i> and <i>Agrobacterium</i>	360 endophytic strains of <i>Pseudomonas</i> and <i>Staphylococcus</i>	Surette <i>et al.</i> , 2003
soybean	35 endophytic bacteria	Hung <i>et al.</i> , 2007

endophytes also influence the growth of plant directly by the production of plant growth promoting traits such as IAA production, Phosphate solubilization, siderophore production, ammonia production, nitrogen fixation antagonism against phytopathogens and indirectly by induced systemic resistance (ISR). Endophytic bacteria colonize an ecological niche similar to that of phytopathogens, which makes them suitable as biocontrol agents (Berg *et al.*, 2005a). Endophytic microorganisms control plant pathogens (Sturz & Matheson, 1996; Duijff *et al.*, 1997; Krishnamurthy & Gnanamanickam, 1997), insects (Azevedo *et al.*, 2000) and nematodes (Hallmann *et al.*, 1997, 1998) through endophyte-mediated *de novo* synthesis of novel compounds and antifungal metabolites. Endophytes can also accelerate seedling emergence, promote plant establishment under adverse conditions (Chanway, 1997) and enhance plant growth (Bent & Chanway, 1998).

Bacterial endophytes stimulate the growth of host plant by nitrogen fixation, enhancement in the availability of minerals and the production of phytohormones (Hurek *et al.* 2002; Iniguez *et al.* 2004; Sevilla *et al.* 2001). Endophyte mediated *de novo* synthesis of antifungal or antibacterial metabolites, siderophores and competition for nutrients induce systematic resistance in the host to check the progress of disease (Sessitsch *et al.* 2002a; Sturz *et al.* 2000).

4. Endophytic bacteria as bio fertilizers

Research has been revealed that endophyte increase plant growth through the improved cycling of nutrients and minerals such as phosphate solubilisation (Verma *et al.*, 2001; Wakelin *et al.*, 2004), indole acetic acid production (Lee *et al.*, 2004) production of siderophore (Costa and Loper, 1994) and supply of essential vitamins to plants (Pirttila *et al.*, 2004). Compant *et al.* (2005) reported that endophytes also influence other beneficial effects of host include osmotic

adjustment, stomatal regulation, modification of root morphology, enhanced uptake of minerals and alteration of nitrogen accumulation and metabolism. Adhikari *et al.* (2001) reported that endophytic bacterial strains are potential in controlling the seedling disease of rice and promote growth in rice. Application of endophytic bacterial strains significantly increased the growth parameters viz., pseudostem height, girth, number of leaves and physiological parameters viz., chlorophyll stability index, stomatal resistance and transpiration in banana plants both under greenhouse and field conditions (Harish, 2005).

4.1. IAA production

According to earlier studies IAA production by endophytes can vary among different species and isolates and it is also influenced by culture condition, growth stage and substrate availability. Out of 65 endophytes of soybean, 15 isolates were positive for IAA production produced more than 25 µg/ml of IAA and *Acetobacter diazotrophicus* and *Herbaspirillum seropedicae* found to produce IAA in chemically defined culture media. Seven out of 10 endophytic isolates of *Typha australis* were positive for IAA production (Hung and Annapurna 2004; Chen *et al.*, 1998; Jha and Kumar, 2007). Two bacterial endophytes of *Capsicum annum* L. also reported to show plant growth promotion and defense against phytopathogens along with IAA production. Long *et al.* (2008) reported 1.1 to 154 µg/ml of IAA production by the endophytic bacteria isolate from *Solanum nigrum*. Gangawar and Kaur (2009) reported 15 endophytic isolates of sugarcane produced 4 to 19.3 µg/ml of IAA. Chilli endophytes are observed to be more potential in IAA production than sugarcane and similar to *Solanum nigrum* reported (Sudhir 2014). Amrutha *et al.* (2012) reported that *Pseudomonas aureginosa* CEF3, *Bacillus sp* CEF19, *Curtobacterium oceanosedimentum* AVSCE3 and *Bacillus cereus* AVSCE 5

isolated from different parts of chilli produced 23µg/ml, 21µg/ml, 111.5µg/ml and 125µg/ml of IAA, respectively. It was much higher than that of found in other reports (Long et al. 2008).

Harish et al., (2008) assessed the plant growth promotion efficacy of 45 endophytic bacteria isolated from corn and root of banana. 12 strains isolated from gingseng plant, endophytic *P. fluorescens* WCS365 as biocontrol s bacteria isolated from *Lycopersicon esculentum* produced significant amounts of IAA (Thamizh Vendan et al., 2010, Patel et al., 2012). Three strains isolated from sugar beet roots produced indole-3-acetic acid (IAA) promote plant growth significantly increased plant height, fresh and dry weights and number of leaves per plant (Long et al., 2008; Yingwu Shi et al., 2009). Vetrivelkalai et al., 2010 also reported significant enhancement in the germination percentage, shoot and root length and vigour index of bhendi seedlings by seed bacterization.

4.2. Phosphate solubilization

Earlier reports revealed endophytic bacteria also solubilize phosphate from organic or inorganic bound phosphates and type of organic acid produced during phosphate solubilization depends on the carbon source utilized as substrate. Highest P solubilization has been observed when glucose, sucrose or galactose has been used as sole carbon source in the medium (Khan et al., 2009; Park et al., 2010). Endophytic bacteria able to solubilize inorganic phosphate and extracellular tricalcium phosphate effectively in presence of glucose (Kuklinsky - Sobral et al., 2004; Long et al., 2008; Thamizh Vendan et al., 2010; Patel et al., 2012). Endophytic *Bacillus*, *Pseudomonas*, *Klebsiella* and *Acinetobacter* are also reported as potential phosphate solubilizers (Huang et al., 2010). Endophytic *Bacillus cereus* and *B. megaterium* isolated of Ginseng plant also showed significantly high P solubilization (ThamizhVendan et al., 2010). Endophytic and rhizosphere

Pseudomonas spp. Isolated from Serbia able to solubilize TCP (Stajković et al., 2011; Djuric et al., 2011; Josic et al., 2012b). Amrutha et al. (2012) reported that *Pseudomonas aureginosa* CEFR3 (198ppm/ml), *Bacillus* sp CEFR19 (1354ppm/ml) isolated from ripened fruit and isolated from green fruit and *Bacillus cereus* AVSCE 5(137ppm/ml) isolated from leaf of chilli are able to solubilize inorganic phosphate efficiently. Increase in the yield of canola by endophytic *Bacillus* sp. was reported by de Freitas et al. (1997). Sundara et al. (2002) reported that enhancement in available phosphorus and yield of sugarcane by application endophytic bacteria. *Pseudomonas* spp. are able to increase the growth and phosphorus content of maize by endophytes (Vyas and Gulatti, 2009).

4.3. Siderophore

Researchers reported that endophytic fungal siderophore have lower affinity than bacteria to sequester iron and deprive pathogenic fungi (Whipps, 2001; Loper and Henkels 1999). Endophytic bacterial isolates associated with hyacinth and Genseng plants produce siderophore (Jafra et al., 2009; Thamizh Vendan et al., 2010). Rajkumar et al., (2010) reported that the siderophore play a pivotal role in nitrogen fixation under iron deficiency.

4.4. Nitrogen fixation

According to earlier studies endophytic bacteria better express their nitrogen fixation potential inside plant tissues due to the lower competition for nutrients and protection against high levels of O₂ present on the root surface. Many diazotrophic bacteria are able to establish a symbiotic relationship with plants for biological nitrogen fixation (Robson et al., 1986; Chisnell et al., 1988; Dekas et al., 2009). Earlier studies revealed that endophytic diazotrophs constitute only a small proportion of total endophytic bacteria include *Azospirillum lipoferum*, *Klebsiella pnemoniae* and *Azorhizobium caulinadans* (Schloter et al., 1994; Bar-

raquio *et al.*, 1997; Martínez *et al.*, 2003). Unlike symbiotic diazotrophes, endophytic bacteria are unable to form nodules. *Gluconacetobacter diazotrophicus* was identified as first N₂-fixing endophytic bacteria associated with sugarcane stem (Cavalcante and Dobereiner, 1988) and confirmed by other scientists in USA, UK, and Germany and two more N₂-fixing endophytes *Herbaspirillum seropedicae* and *H. rubrisubalbicans* were reported by Boddey *et al.*, (1995). James (2000) reported *Herbaspirillum sp.* as endophytic diazotroph in sugarcane and rice. *Azoarcus sp.* in rice and Kallar grass and endophytic *Klebsiella sp.* Kp342 strain of wheat identified as nitrogen fixers (Iniguez *et al.*, 2004). Silva-Froufe (2009) reported *Glucanoacetobacter diazotrophicus* as endophytic diazotrophic bacteria in sugarcane, sweet potato, and pineapple. Endophytic *Bacillus* species of soybean nodule showed potential N fixing and reported to improve root growth and function, (Bai *et al.*, 2002; Asis *et al.*, 2004; Matiru and Dakora, 2004). 23 endophytic bacteria are identified as potential ammonia producers in chilli (Amrutha *et al.*, 2012) and reported that *Pseudomonas*, *Curtobacterium* and *Bacillus sp* isolated from chilli were found to be maximum producers of Ammonia.

4.5. Volatile compounds

Earlier literature revealed that endophytic bacteria can produce a wide range of volatiles. Biological function of most of these volatiles is not yet understood. It is assumed that volatile compounds involved in a number of processes including cell-cell signaling, inter-species signaling, promote plant growth and act as microbial inhibiting agents (Wheatley, 2002; Vesperman *et al.*, 2007; Kai *et al.*, 2009). Ryu *et al.*, (2003) reported that *Bacillus sp.* produce 2, 3 butanediol and acetoin and promote plant growth in *Arabidopsis thaliana*. 38 volatile compounds were reported from rhizobacteria (Farag *et al.*, 2006). Blom *et al.* (2011) screened 42 strains in four different growth media

and studied growth response of *A. thaliana*. Each strain showed significant volatile-mediated plant growth modulation and also reported that *Burkholderia pyrrocinia* as significant plant growth-promoter. The volatiles indole, 1-hexanol and pentadecane promotes growth only under stress conditions

5. Endophytes as biological control agents (BCA)

Endophytes are potential biocontrol agents like other biocontrol agents such as associative nitrogen fixing PGPB on sugarcane (Boddy, 2003) or *Burkholderia phytofirmans* PsJN, non-symbiotic endophytic bacteria (Sharma and Nowak, 1998) endophyte holds potential of BCA may be due to self-perpetuating nature of endophytes inside the host by colonization and being transfer to progeny (Boddy, 2003). According to Backman *et al.* (1997), the effectiveness of endophytes as biological control agents (BCA) is dependent on many factors. These factors include: host specificity, the population dynamics, pattern of host colonization, ability to move within host tissues and the ability to induce systemic resistance. Certain endophytic bacteria trigger induced systemic resistance (ISR) which is phenotypically similar to systemic-acquired resistance (SAR). SAR develops when plants successfully activate their defense mechanism in response to primary infection by a pathogen and induces a hypersensitive reaction in the form of a local necrotic lesion of brown desiccated tissue (van Loon *et al.*, 1998). ISR is effective against different types of pathogens but differs from SAR because in ISR the inducing bacterium does not cause visible symptoms on the host plant (van Loon *et al.*, 1998).

The first record of an endophyte affecting a plant disease was reported by Shimanuki (1987) who showed that *Phleum pratense* plants infected with the *Epichloe typhina* were resistant to the fungus *Cladosporium phlei*. In some cas-

es, endophytes also accelerate seedling emergence and promote plant establishment under adverse conditions and enhance plant growth and development (Pillay and Nowak, 1997). Several endophytic bacterial species including *Achromobacter* sp., *Acinetobacter baumannii*, *A. lwoffii*, *Alcaligenes*, *Moraxella* sp., *Alcaligenes* sp., *Arthrobacter* sp., *Bacillus* sp., *Burkholderia cepacia*, *Citrobacter freundii*, *Corynebacterium* sp., *Curtobacterium flaccumfaciens*, *Enterobacter cloacae*, *E. aerogenes*, *Methylobacterium extorquens*, *Pantoea agglomerans*, *Pseudomonas aeruginosa*, and *Pseudomonas* sp. isolated from the xylem of lemon roots (*Citrus jambhiri*) have been reported as antagonistic bacteria against root phytopathogens (Araújo et al., 2001; Lima et al., 1994).

Cabbage treated with endophytes did not reach the economic threshold for the disease approximately 50 days after inoculation with *Xanthomonas campestris* pv. *Campestris* due to the induction of defense mechanisms (Jetiyanon, 1994). Several bacterial endophytes have been reported to support growth and improve the health of plants (Hallmann et al., 1997). *Erwinia caratovora*, for example, is inhibited by numerous endophytic bacteria, including several strains of *Pseudomonas* sp., *Curtobacterium luteum*, and *Pantoea agglomerans* (Sturz et al., 1999).

Wilhelm et al. (1997) demonstrated that *Bacillus subtilis* strains isolated chestnut trees exhibit antifungal effects against *Cryphonectria parasitica* causing chestnut blight. The ability of endophytic bacteria to inhibit pathogen has been decreased in potato tubers due to deep colonization interior to the plant host (Struz et al., 1999). Barka et al. (2002) demonstrated the ability endophytes to colonize in divergent hosts. *Pseudomonas* sp. an onion endophyte colonized in grape vine inhibited *Botrytis cinerea* Pers. and promoted growth in grapevines. Colonization of multiple hosts has also been observed with other species of endophytes such as *Pseudomonas putida* 89B-27 and

Serratia marcescens 90-166 reduced Cucumber Mosaic Virus (CaMV) in tomatoes and cucumbers (Raupach et al., 1996), anthracnose and *Fusarium* wilt in cucumber (Liu et al., 1995).

Sixty one (61) endophytic bacteria isolated from potato stem tissues were identified as effective biocontrol agents against *Clavibacter michiganensis* subsp. *Sepedonicus* (Sturz et al., 1999). *Bacillus mycoides* BacJ and *Bacillus pumilis* 203-7 isolates from different host plants suppressed *Cercospora* leaf spot in sugar beet (Bargabus et al., 2002: 2004). Araujo et al. (2002) reported *Curtobacterium flaccumfaciens*, citrus endophyte help citrus plants to better resist against the pathogenic infection of *Xylella fastidiosa*. Endophytes isolated from potato plants produce antibiotics and siderophore and showed antagonism against fungal pathogens (Berg et al., 2005a; Sessitsch et al., 2004) and bacterial pathogens *Erwinia* and *Xanthomonas* (Sessitsch et al., 2004). Of 2,648 bacterial isolates from the rhizosphere, phyllosphere, endosphere, and endorhiza, only *Serratia plymuthica* root endophyte was a highly effective against *Xanthomonas* sp. in Brassica seeds (Berg et al., 2005b).

Endophytic actinobacteria also show effective antagonism against the pathogenic fungus *Gaeumannomyces graminis* of wheat (Coombs et al. 2004).. A number of endophytic actinobacteria isolated by culture dependent methods belong to the genera of *Streptomyces*, *Microbispora*, *Micromonospora*, and *No-cardioides* (Coombs and Franco, 2003a) were capable of suppressing *Rizoctonia solani*, *Pythium* spp., and *Gaeumannomyces graminis* var *tritici*, fungal pathogens of wheat *in vitro* and *in planta*, (Coombs et al., 2004).

Aravind et al. (2009) reported against. Native endophytes IISRBP 35, IISRBP 25 and IISRBP 17 isolated from black pepper exhibited 70% disease suppression of *Phytophthora capsici* in black pepper in greenhouse trials. The intimate relationship between endophytic bacteria and

their hosts make the endophytes as natural candidates for selection as biocontrol agents with high level of competition (Chen *et al.*, 1995; Van Buren *et al.*, 1993).

Twenty two (22) endophytic bacteria isolated from different parts of chilli plant showed antagonism against *Colletotrichum capsici*, *C. gloeosporioides* and *C. acutatum* (Amrutha *et al.*, 2014). Antagonism of endophytic bacteria against *Clavibacter michiganensis* subsp. *sepedonicum* cause rot on tomato (Van Buren *et al.*, 1993), *Pseudomonas chlororaphis*, *P. fluorescens*, *P. graminis*, *P. putida*, *P. tolaasii* and *P. veronii* against bacterial pathogens (Chen *et al.*, 1995; Adhikari *et al.*, 2001) Invitro inhibition of various fungal pathogens by *Bacillus subtilis* ME488nd (Chung *et al.*, 2008) have also been reported.

6. Induced systemic resistance (ISR)

Endophytes have a natural and intimate association with plants because the internal tissues of plant provide relatively uniform and protected environment and favors endophytic bacteria to be a potential agent of ISR (Chen *et al.* 1995). Viswanathan (1999) reported that application of endophytic strain is more beneficial in vegetatively propagated crops like banana, sugarcane and tapioca for inducing systemic resistance and also reported that endophytic *P. fluorescens* strain EP1 isolated from stalk tissues of sugarcane induced systemic resistance against red rot caused by *Colletotrichum falcatum* (Viswanathan and Samiyappan, 1999a). van Loon (2007) reported that the phenomenon of ISR has been noted to be exhibited both associative and endophytic bacteria. ISR triggered by endophytes fortifies plant cell wall strength, alters host physiology and metabolic responses and enhance synthesis of plant defense chemicals such as phenolic compounds, pathogenicity related protein (PR-1, PR-2, PR-5), ROS enzymes (chitinases, peroxidases, polyphenyloxidase, phenyl alanine

ammonia lyase, , oxidase and/or chalcone synthase) and phytoalexins to protect the host plant from future infections (Nowak and Shulaev, 2003). Elicitors include lipopolysaccharides, flagella, siderophore, antibiotics, VOCs or quorum-sensing signals produced by endophytic bacteria elicit ISR in plants (Van Loon, 2007). ISR activated by PGPB is mediated by jasmonate or ethylene in majority of plants. Thickening of the outer tangential and outermost part of the radial side of the first layer of cortical cell walls was also noticed in tomato roots with the treatment of endophytic *P. fluorescens* WCS417 (Duijff *et al.*, 1997).

Application of endophytic bacteria by stem injection in cotton plants reduced the root rot caused by *Rhizoctonia solani* and vascular wilt caused by *F. oxysporum* f. sp. *vasinfectum* (Chen *et al.*, 1995). Pleban *et al.* (1995) reported that endophytic bacteria move upward and downward from the point of application before colonizing the internal tissues and check the entry of *F. solani* in cotton and *Sclerotium rolfsii* in beans. Application of *Pseudomonas fluorescence* prevented the entry *Pythium ultimum* in the roots of pea, *P. fluorescence* restrict the growth of *Fusarium oxysporum* f. sp. *pisii* and *P. ultimum* in pea plant (Benhamou *et al.*, 1996b, 1998). *Pseudomonas* strain 63-28 also induced resistant in tomato against *F. oxysporum* f. sp. *radicislycopersici* (M'Piga *et al.*, 1997). Inoculation of endophytic *Pseudomonas denitrificans* strain 1-15 and *P. putida* strain 5-48 protected the oak tree against *Ceratocystis fagacearum* (Brooks *et al.*, 1994). Recent reports have been reviewed mechanisms of ISR induced by endophytic *Pseudomonas* (Jankiewicz and Koltonowicz, 2012). *Bacillus cereus* AR156 in *A. thaliana* (Niu *et al.*, 2011) and different endophytic *Bacillus* sp. from vegetable crops induced systemic resistance. Combination of three endophytic isolates resulted in significant growth promotion and ISR in tomato, bell pepper, cucumber and tobacco (Kloepper *et al.*, 2004). Mixed formu-

lation of *B. subtilis* GB03, *B. amyloliquefaciens* IN937a and *B. subtilis* IN937b together with chitosan in tomato and different endophytic *bacillus* sp. isolated from vegetable crops induced systemic resistance in *Theobroma cacao* seedlings. Harish *et al.* (2008) reported mixed formulation of rhizobacterial and endophytic bacterial (EPB5 + EPB22 + Pf1 + CHA0) was significantly effective in suppressing Banana Bunchy top virus under field conditions.

7. Perspectives

Microorganisms with phytopathogenic antagonism act as Biocontrol agents (BCA). Most of the biocontrol agents have not fulfilled their initial promise because of poor rhizosphere competence. The failure of BCA being attributed the difficulties in long-term culture. It would obviate the need for selecting bacterial types with high levels of rhizosphere competence and successful seed or root bacterization treatments before or at planting (Schroth *et al.*, 1984; Weller, 1988). The intimate relationship between endophytic bacteria and their hosts make the endophytes as natural candidates as biocontrol agents with high level of competence (Chen *et al.*, 1995; Van Buren *et al.*, 1993).

Selection of endophytic bacteria that can elicit plant growth promotion and ISR in plants and research on such endophytes in understanding plant responses that occur during the signal transduction pathways that culminate in disease protection is essential to delineate the pathosystems. In many cases, elicitation of ISR by endophytic bacilli is associated with increased plant growth, and the relationship between ISR and growth promotion should be further investigated. Elucidation of specific bacterial determinants that account for elicitation of ISR is just beginning, and further work is needed to understand why one strain of a given bacterial species can elicit ISR while another strain of the same species cannot. The

principle behind the plant and endophytic interaction to elicit ISR or plant growth promotion and development of endophytic bioformulations will be an index of growing scientific knowledge in agriculture and horticulture.

8. Concluding remarks

According to the research conducted so far, the use of chemical fertilization is necessary because biological fertilization has not yet proven to be good enough in providing complete plant nutrient requirements. It has been indicated that about less than 50% of chemical fertilizers is absorbed by plant and the rest would not be accessible by plant as it is subjected to leaching, runoff, and emission from the soil surface. Hence, the use of biological fertilizers as supplementary fertilization to chemical fertilization is necessary with the above mentioned advantages. Accordingly, the right and proper application of chemical and biological fertilization is very much dependent on realizing the interactions between soil, plant and microorganisms. Endophytic bacteria a big help to plant and the environment as they own great abilities that collectively enhance plant growth and ISR. Among such abilities, enhanced nutrient uptake by plant is also of great importance; in the presence of soil microbes, plant absorbs higher amounts of nutrients and less risk of environmental pollution is likely. Some of the most important functions of endophytic bacteria were reviewed in this article. However, the particular emphasis has been on the use of endophytic bacteria for biological fertilization and biological control of phytopathogens. PGPR bio-fertilization is a very common method because of its rapid effects on plant growth and yield production. But there are issues regarding the use of PGPR fertilizers, as their competence in soil and interaction with plant vary with the rhizosphere environment. Since the endophyte is metabolically acclimatized to the host plant with mutual-

ism indicate the contribution of endophytes as biological fertilizer is better option in comparison to other fertilizers. For the development of efficient endophytic bacterial biofertilizers, the microbes must be properly selected, combined and formulated with respect to the agricultural and environmental conditions.

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Microbial Metabolic Engineering: A Key Technology to Deal with Global Climate and Environmental Challenges

Meerza Abdul Razak¹, Pathan Shajahan Begum², Senthilkumar Rajagopal³ *

¹Department of Biotechnology, Rayalaseema University, Kurnool, Andhra Pradesh, India; ²Department of Zoology, KVR Women's degree college, Kurnool, Andhra Pradesh, India; ³Department of Biochemistry, Rayalaseema University, Kurnool, Andhra Pradesh, India; *Correspondence: senthilanal@yahoo.com; Tel: +91 9566860390

Abstract: Global climate change and green house effects are very serious and controversial problems that have severe negative impacts on environment, society, energy industry and government policies and sustainability. Global environment and climate challenges are directly connected to the accumulation of green house gases which has caused concerns related to the usage of traditional and fossil fuels as the key energy resource. To mitigate climate and environment changes, one solution is to utilize the potential of metabolic engineering of microbes for biofuels production from renewable sources. For long-term economic sustainability of the energy, industries and transportation sectors should adopt renewable and sustainable fuels produced by metabolically engineered microorganisms. The biofuels produced from renewable sources by metabolically engineered microbes carry good energy contents with minimal emission of greenhouse gases and causes minimal impact on the environment, food chain, water supply and land use. Toxic organic and inorganic chemicals are also one of the main reasons for environment contamination and also present major risk for climate change. Avoiding of upcoming contamination from these chemicals poses a huge technical challenge. Currently, metabolically engineered microbes have been explored only for selective and high capacity bioremediation of heavy toxic metals and chemicals. This chapter will shed light on current trend and developments in metabolic engineering of microbes for biofuel and bio-based chemicals production from renewable resources. This chapter also highlights the potential of metabolically engineered microbes for bioremediation, a possible futuristic solution for sustainable development for energy and reduction of global climate change and green house effects.

Keywords: Biofuels; bioremediation; metabolic engineering; synthetic biology; systems biology

1. Introduction

Since the past some decades, constantly increasing greenhouse gases in environment such as carbon dioxide, nitrous oxide, methane have been linked to global environmental and climate concerns (O'Neill and Oppenheimer, 2002; Stocker, 2013). Some of the effects of global climate and environment change are abolition of coral reef, rising of sea

level and weakening of thermohaline circulation. The atmospheric carbon dioxide concentration is 400 parts per million (ppm) and the carbon dioxide released from fossil fuels worldwide is 7 Gt of carbon per year (Pacala and Socolow, 2004; Lewis and Nocera, 2006). If the present upward trend of carbon dioxide continues, by the end of year 2050 the carbon dioxide release rate will be doubled. It is estimated that the carbon diox-

ide concentration will reach to 500 ppm by doubling of the carbon dioxide emission rate without remediation. The carbon dioxide concentration of 500 ppm will lead to a global warming of around 2°C above the level in year 1900 (Pacala and Socolow, 2004). This level of increase in temperature would raise the threat of disintegration of the West Antarctic Ice Sheet (WAIS) along with other negative effects. It is estimated that increase in temperature by 2°C would lead to disruptive rise of sea level by 4–6 meters (Stocker, 2013).

The atmospheric CO₂ concentration, CO₂ emission and global temperature are having severe negative effects on the environment, climate, economy and society and we need to deal with it. To avoid the rise in the temperature, it is necessary that we should reduce the carbon emission from the fossil fuel usage and increase the sources of renewable energy and remove the toxic chemicals and metals present in the environment. When compared with different renewable energy sources, biofuels are well-suited with present infrastructure and have an advanced energy density. Thus there has been much curiosity in establishing biorefineries for the production of fuels and chemicals from renewable resources.

We are in the modern age of microbial metabolic engineering which comprises of progressively more efforts at cell, and pathway design. One of the reasons for the more success of microbial metabolic engineering is development of additional “omics” tools which provide both temporally and spatially analyzing opportunity for cellular systems at the level of protein, metabolite, RNA and DNA (Peralta-Yahya *et al.*, 2012). Microbial metabolic engineering have been evolved to solve crucial international problems such as global warming, bioremediation, food and human health. If properly utilized microbial metabolic engineering can play an important role in facing the global challenges. By development of novel technological innovation

in combination with rising global crises provides the platform for microbial metabolic engineering applications and innovation on a large scale. Scientists from industries and academics strongly believe that microbial metabolic engineering in combination with other technologies such as synthetic biology and systems biology can help to solve the growing concerns of climate and environment (Zhang *et al.*, 2011).

From the beginning microbial metabolic engineering had aimed the production of fuels and chemicals as chief goals of the rising field. The metabolic engineering of yeast *Saccharomyces cerevisiae* and *E. coli* are the classic examples of metabolic application in the field of biofuel production (Kuyper *et al.*, 2003, Bro *et al.*, 2006, Ingram *et al.*, 1987). Microbial metabolic engineering applications have expanded in the past few years because of the growing attention on biofuels and chemical production through biomass conversion. There are many reports stating that the combination of microbial metabolic engineering along with combinatorial approaches supported by high throughput systems had given good results in biofuel production. The key advantage of utilizing microorganism for the production of biofuels from renewable sources is the metabolic diversity of fungi, algae and bacteria facilitate us the use of diverse substrates as the starting point for biofuel generation.

Bioremediation is a very cost effective and eco-friendly method and is steadily making inroads for environmental clean-up applications. Bioremediation depends on enhanced detoxification and degradation of toxic metals and degradation of toxic pollutants either through enzymatic transformation or intracellular accumulation to less or non-toxic compounds. There are several physiochemical processes for treating toxic pollutants in environment, but these processes are non specific, very costly and sometimes they may introduce secondary contamination. Microbes naturally have the

capability to transform, degrade and chelate several toxic chemicals. But the microbial bioremediation process is having relative slow transformation rates. By metabolically engineering microbes it is possible to remove the toxic inorganic and organic chemicals from the environment (Shailendra *et al.*, 2008). The better understanding of microbes' natural transformation ability at genetic level and advance of novel genetic tools are very essential for metabolic engineering of microorganism for bioremediation. There are several metabolically engineered microorganisms with superior biotransformation capacity and more accumulation of toxic wastes. In this chapter we discuss metabolic engineering strategies and successful examples of metabolically engineered microorganisms for production of biofuels and chemicals and for bioremediation (Brar *et al.*, 2006).

2. Biofuels production by metabolically engineered microorganisms

With the increasing costs of energy and the challenges of global warming that arise due to the usage of petroleum based feedstock, the scientific community throughout the globe is searching for energy substitutes without adding up to the existing carbon footprint. Biofuels can be an exciting substitute to solve both the climate and environmental issues since they are produced from the renewable resources. Microbial production of hydrogen as future fuel is a hopeful possibility as an alternative for petroleum based fuels. Hydrogen is a more energy dense source and its conversion to power or heat is very simple and clean. Hydrogen when combusted with oxygen only H₂O is formed without the formation of toxic pollutants (Kim and Lee 2010, Panagiotopoulos *et al.*, 2009).

2.1. Hydrogen production

Large number of microbes such as *Sporoacetigenium mesophilum*, *Clostridi-*

um beijerinckii and *Thermoanaerobacterium thermosaccharolyticum* has been used for the hydrogen production. The drawback of these microbes is low production of hydrogen (Cai *et al.*, 2011, Oh *et al.*, 2011). Kim *et al.* overcome the major obstacle of low hydrogen production by metabolically engineered *E. coli* strains (Kim *et al.*, 2009). In one of the investigation a high volumetric productivity of 2.4 H₂/L/h was produced using immobilized cells of a metabolically engineered *E. coli* which had deletion mutation (Seol *et al.*, 2011). Even though, biological hydrogen production was considerably increased by metabolically engineered *E. coli* strain, several vital obstacles involved in the productivity, yield and metabolic robustness are still not up to the mark that would permit commercialization.

2.2. Bioethanol production

Bioethanol is the major renewable liquid energy source comprising 90% of the global world Biofuel market. It is estimated that annual production of bioethanol throughout the world is more than 105 billion liters. Most of the Bioethanol production is by yeast and it is based on the sugarcane and starch, this type of production competes with feed and food (Geddes *et al.*, 2011b). In one of the study *E. coli* was metabolically engineered to efficiently convert glycerol to ethanol. This strain was able to convert 40 g/L glycerol to ethanol in 48 h with 90% of the ethanol yield (Trinh and Srienc, 2009). By introducing the *adhB* and *pdc* genes from *Zymomonas mobilis* which encodes alcohol dehydrogenase and pyruvate decarboxylase into *E. coli* redirected the carbon flux into the ethanol production and the obtained metabolically engineered *E. coli* produced ethanol upto 1.28% (v/v) using xylose as carbon source within 36 hour fermentation (Sanny *et al.*, 2010). Metabolic engineering approaches were implemented to engineer *E. coli* for ethanol production from mixed

sugars, glucose and xylose (Sanny *et al.*, 2010, Wang *et al.*, 2008).

2.3. Isopropanol production

E. coli do not have some of the necessary pathways which are very much necessary pathways for the production of advanced fuels, metabolic engineering has provided the chance to produce non-traditional biofuels by the construction of non native biosynthesis pathways (Atsumi *et al.*, 2008b). The microorganisms such as *Clostridium* can naturally produce Isopropanol which is one of the secondary alcohols. Isopropanol has several diverse applications. In *Clostridium* several attempts have been made to enhance the production ability, but product inhibition and low titer. As a result, metabolic engineering of *E. coli* for Isopropanol production become a promising substitute for industrial production of Isopropanol. Metabolically engineered strain of *E. coli* was constructed that produced a 13.6 g/L isopropanol from glucose under vigorous aerobic culture conditions (Jojima *et al.*, 2008). Isobutanol has same physical properties like isopropanol, except it has higher octane number. Metabolic engineering of *E. coli* resulted in the production of isobutanol using non-fermentative pathways through 2-ketoisovalerate as precursor.

2.4. 1- butanol and 1- Propanol production

1-Butanol is very attractive and alternative biofuel with high energy density and good compatibility with the ability to completely substitute gasoline. Generally, *C. acetobutylicum* produces 1-butanol along with butyrate, ethanol and acetone. But due to absence of genetic information, complex physiology and unavailability of genetic tools the *C. acetobutylicum* had not been improved to the level of usage in large scale industrial production. The complete information of the 1-butanol metabolic pathway, it become possible to construction of butanol pathway in *E. coli*. Atsumi *et al.* devel-

oped an *E. coli* strain which can fermentatively produce 1- butanol by transferring a group of six genes from *C. acetobutylicum* 1- butanol pathway and removal of the competing pathway. This metabolically engineered *E. coli* strain produced 552 mg/L of 1- butanol under semi-anaerobic conditions from a rich medium (Atsumi and Liao, 2008a). 1- Propanol is universal solvent with several industrial applications which can be converted to propylene and diesel and it is a promising gasoline substitute. The wild strain organisms cannot produce the 1-propanol is considerable amounts (Shen and Liao, 2008). 2-ketobutyrate is a precursor of 1-propanol and isoleucine. It is also can be converted to 2-methy 1-butanol and 1- butanol through several multi steps enzymes reactions. *E. coli* was metabolically engineered to produce 1-propanol and 1-butanol through 2-ketobutyrate (Shen and Liao, 2008).

Metabolically engineered strain was developed by overexpression of the genes such as thrAfbBC, leuABCD, ilvA from *E. coli*, kld from *L. lactis* and adh2 from *S. cerevisiae* followed by deletion of the competing genes such as adhE, ilvB, metA and tdh. This engineered strain produced 2 g/L propanol and butanol in 1: 1 ratio. The drawback this developed strain is it production of unwanted fermentative by products. In another study, a more direct pathway to produce 2-ketobutyrate through citramalate pathway was engineered in *Methanococcus jannaschii* by directed evolution to produce 1-butanol and 1- propanol. CimA specific activity was enhanced by the DNA shuffling and error prone PCR in *E. coli* strain. This *E. coli* strain with enhanced CimA activity produced more than 524 mg/L and 3.5g/L propanol. The production of other side products is drawback of this strain.

2.5. 2-Methyl-1-butanol and 3-methyl-1-butanol production

The five carbon alcohols such as 3-methyl-1-butanol and 2-Methyl-1-butanol have characteristics like high energy density and lower vapor pressure. When compared with ethanol, 3-methyl-1-butanol and 2-Methyl-1-butanol are more suitable to replace gasoline and more well-suited for the present fuel infrastructure. 3-methyl-1-butanol and 2-Methyl-1-butanol can be naturally produced by as by-products and some metabolic approaches have been tried to enhance the production of 2-Methyl-1-butanol in *S. cerevisiae* (Abe and Horikoshi, 2005). Table 1 illustrates the biofuels production by metabolically engineered *E. coli*.

3. Bioremediation by metabolically engineered microorganisms

3.1. Bioremediation of organic pollutants

The extensive use of extremely toxic compound organophosphates (OPs) in agriculture as pesticide had led to a serious environmental pollution. Organophosphates are used in the insecticides; generally organophosphates are in the form of phosphoric acid. The bacteria growing in the soil had naturally acquired the ability to degrade organophosphates with the help of enzyme called organophosphate hydrolase (McDaniel *et al.*, 1988; Kulkarni and Chaudhari 2006). P-O linkage is hydrolysed by the organophosphate hydrolase, releasing p-nitrophenol as leaving group. As the toxicity of organophosphates is extensively diminished by hydrolysis of phosphoester bonds, several scientists have concentrated on the primary hydrolysis by organophosphate hydrolase. Even though organophosphates are transformed into dialkyl phosphates and p-nitrophenol (PNP) by initial hydrolysis. Still these degraded products are resistant to biodegradation and they are toxic. One solution to remove even

the less toxic dialkyl phosphates and p-nitrophenol is by metabolic engineering of microbes with the required degradation pathways (Cook *et al.*, 1980; de la Pena Mattozzi *et al.*, 2006).

The p-nitrophenol can be degraded by metabolically engineered *P. putida* and *Moraxella* species. In *Moraxella* species organophosphate hydrolase was expressed on the cell surfaces and this strain showed to degrade paraoxon, parathion and methyl parathion and their hydrolysis product p-nitrophenol. In *P. putida* strain, natural p-nitrophenol degrading operon was introduced. A synthetic operon for expression of alkaline phosphatase (PhoA), phosphodiesterase (Pde) and organophosphate hydrolase was introduced to improve the diethyl phosphate (DEP) mineralization and hydrolysis of organophosphates. The resulting metabolically engineered *P. putida* strain was able to totally degrade p-nitrophenol in 78 hours, paraoxon in 24 hours and diethyl phosphate within 142 hours (de la Pena Mattozzi, *et al.*, 2006). This research study presents an effective novel approach to create an artificial metabolite pathway for total mineralization.

The present hydrodesulfurization method for decreasing the level of organic sulfur compounds is not capable to please the strict government regulations. The present advances in desulfurization have therefore been focused on biological processes using microbes. Dibenzothiophene which is a popular organosulfur compound is not removed by the hydrodesulfurization from fossil fuels. The desulfurization of dibenzothiophene requires a group of enzymes (Kilbane, 2006). Dibenzothiophene monooxygenase (DszC) is the first enzyme that converts dibenzothiophene to dibenzothiophene sulfone. The dibenzothiophene sulfone is converted into 2-hydroxybiphenyl-2-sulfinate by the catalysis of dibenzothiophene-5,5-dioxide monooxygenase. 2-hydroxybiphenyl-2-sulfinate is converted

Table 1: Biofuels produced by metabolically engineered *E. coli*

Biofuels	Fermentation process	Engineering strategy	Carbon source	Titer and yield	Industrial applications
Hydrogen	Immobilized recombinant cells	Deletion of negative regulator and competing carbon metabolic pathways	Formate	1.0 mol H ₂ /mol formate; 2.4 l H ₂ /L/ha; formate	Fuel and energy carrier
Bioethanol	10-L bioreactor batch anaerobic cultivation	Minimized metabolic functionality for conversion of xylose and glucose into ethanol by multiple-gene knockout	Xylose and glucose	38.81 g/L; 0.49 g/g glucose or xylose	Fuel, solvent, food, beverage
1-Propanol	Shake flasks and IPTG induction	Improving specific activity and releasing feedback inhibition of the key enzyme by directed evolution	Glucose	3.5 g/L; 0.049 g/gb	Gasoline additive, allround solvent
1-Butanol	1-L Bioreactor with aerobic–anaerobic dual phase fermentation	Construction of modified clostridial 1-butanol pathway in <i>E. coli</i> strain and enhancement of driving forces	Glucose	30 g/L; 88% of the theoretical yield	Bulk material, gasoline additive or fuel
3-Methyl-1-butanol	Shake flask with two-phase fermentation	Random mutagenesis and selection combined with overexpression of key genes	Glucose	9.5 g/L; 0.11 g/g glucose	Advanced fuel, alternative gasoline
Isopropanol	Fed-batch fermentation, gas-stripping-based recovery process	Heterologous expression of target product pathway from various sources in <i>E. coli</i> host	Glucose	143 g/L; 67.4% (mol/mol)	Biodiesel, precursor of polypropylene
Isobutanol	Screw-cap conical flasks and IPTG induction	Introducing non-fermentative synthetic pathway in <i>E. coli</i> and elimination of pathways competing for pyruvate and cofactors	Glucose	20 g/L; 86% of the theoretical maximum	Gasoline blend stock, precursor of butenes

into 2-hydroxybiphenyl (HBP) and sulfite by the enzyme 2-hydroxybiphenyl-2-
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sulfinate sulfinolyase (Reichmuth *et al.*, 2004). By mutating at the 50 untranslated

region of dibenzothiophene monooxygenase and overexpressing it enhanced the desulfurization rate by 9 fold increase when compared with the unmutated dibenzothiophene monooxygenase (Reichmuth *et al.*, 2004).

Nitrotoluene and nitrobenzene which are extensively used as pesticides, some polymers dyes and explosives are one of the most commonly seen pollutants. Naturally microorganisms can transform nitroaromatic compounds into amines with the help of redox enzymes. But the degradation is very slow because of electron loosing effect of nitro groups and toxicity. Through metabolic engineering and site directed mutagenesis at the position of 258 of 2-nitrotoluene dioxygenase which is responsible for the oxidation of nitrotoluene to 3-methyl catechol and nitrite changed the enantiospecificity and resulted in more degradation of nitroaromatic compounds (Lee *et al.*, 2005). By metabolic engineering of *Sphingobium chlorophenolicum* ATCC 39723 the rapid degradation of another toxic pesticide pentachlorophenol was achieved. Three rounds of genome shuffling in *Sphingobium chlorophenolicum* resulted in a strain with more resistance to pentachlorophenol, increased growth and rapid pentachlorophenol removal (Dai and Copley, 2004).

3.2. Bioremediation of inorganic pollutants

Generally microorganisms when they come in contact with heavy metals, they synthesize metal binding peptides such as metallothionein and phytochelatins. These peptides which are rich in thiol group bind to different heavy metals and by sequestration they reduce the toxicity. Moreover, these peptides produced in different sub cellular locations of microbes have been very useful for them to enhance their metal accumulation ability. *E. coli* was metabolically engineered by over expression of phytochelatin synthase of *Arabidopsis thaliana* which is responsible for synthesis of phytochelatins, this

resulted in 20 fold higher heavy metal accumulation (Sauge-Merle, *et al.*, 2003). Metallothionein and phytochelatins have restriction such as non selective binding to diverse heavy metals. Specific heavy metal transporters support to enhance uptake and accumulation of particular toxic heavy metals. By expression of Cd transporter MntA, selective Cd accumulation can be achieved (Kim, *et al.*, 2005). The *Fucus* Metallothionein which is obtained from the arsenic resistant marine alga *Fucus vesiculosus* is used in metabolic engineering to create superior strains of *E. coli*. The co-expression of specific arsenic transporter and *Fucus* Metallothionein in *E. coli* resulted in 45 fold increase in arsenic accumulation. It is possible that the same strategies can be applied for other heavy metals also. Table 2 elucidates the environmental pollution creating inorganic heavy metals and radionuclides.

The over expression of phytochelatin synthase responsible for enzymatic phytochelatins synthesis by enzymatic method in symbiotic *Rhizobia* bacterium showed good results. From this study it was demonstrated that phytochelatins can be successfully used for heavy metal accumulation, but the limitations in this approach are the minimum supply of the precursor glutathione in phytochelatin production and other heavy metal accumulation. This limitation can be overcome by co-expression of the enzyme phytochelatin synthase and the enzymes responsible for precursor glutathione production Sriprang, *et al.*, (2002). The co-expression of phytochelatin synthase with feedback resistant glutathione synthase resulted in 10 fold increases in phytochelatin production. The overexpression of Cadmium transporter increased the final cadmium accumulation by 31.6 mmol/g dry weight (Kang *et al.*, 2007).

3.3. Bioremediation of radionuclide

In addition to organic and inorganic pollutants, radionuclide contamination occurred through nuclear weapons or

Table 2: Environmental pollution creating inorganic heavy metals and radionuclides

Contaminant	MCLG (mg/L)	MCL (mg/L)	Potential health effects from ingestion of water	Sources of contaminant in drinking water
Arsenic	0	0.010	Skin damage or problems with circulatory systems, and may have increased risk of getting cancer	Erosion of natural deposits; runoff from orchards, runoff from glass and electronic production wastes
Cadmium	0.005	0.005	Kidney damage	Corrosion of galvanized pipes; erosion of natural deposits; discharge from metal refineries; runoff from waste batteries and paints
Lead	0	0.015	Infants and children: delays in physical or mental development; children could show slight deficits in attention span and learning abilities. Adults: kidney problems; high blood pressure	Corrosion of household plumbing systems; erosion of natural deposits
Mercury (inorganic)	0.002	0.002	Kidney damage	Erosion of natural deposits; discharge from refineries and factories; runoff from landfills and croplands
Radium 226/228	0	5 pCi/L	Increased risk of cancer	Erosion of natural deposits
Uranium	0	30 mg/L	Increased risk of cancer, kidney toxicity	Erosion of natural deposits

MCLG: maximum contaminant level goal; MCL: Maximum contaminants limit; Source: EPA safe water

nuclear plant leakage is one of the major environmental problems. Naturally occurring bacteria which are more resistant to radiation are ideal metabolic engineering candidates for enhanced radionuclide removal. Metabolic engineering of thermophilic bacterium *Deinococcus geothermophilus* was done by over expressing of mer operon from *E. coli* coding for Hg²⁺ reduction, which resulted in radiation resistant bacterium (Brim et al., 2003). The

metabolically engineered bacteria *Deinococcus geothermophilus* is able to decrease mercury at higher temperature and ionizing radiation, it is also able to reduce Cr(VI), U(VI) and Fe(III). This study reported the possibility of using metabolically engineered microorganisms for removal of versatile radioactive wastes at high temperatures. In *P. aeruginosa*, radionuclide precipitation can be achieved as metal phosphate by over expressing of

exopolyphosphatases and polyphosphate kinase (Renninger *et al.*, 2004). In radiation resistant bacterium *D. radiodurans*, non-specific phosphatases *phoN* was expressed which resulted bioprecipitation of uranium from dilute nuclear waste (Apukuttan *et al.*, 2006). The enzymes uranyl reductases and chromate are subjected to directed evolution and these engineered enzymes are used for metabolic engineering of *P. putida* and *E. coli*. These metabolically engineered strains of *P. putida* and *E. coli* showed more resistant against radiation and further enhanced the radio-nuclide precipitation efficiency (Barak *et al.*, 2006).

4. Metabolic engineering strategies

Generally the metabolic engineering strategies are based on genetic engineering techniques. Some of the essential requirements for metabolic engineering are 1. Complete information of the biosynthetic pathway of the interested chemical. 2. Particular of the genes coding related enzyme. 3. Regulating factors of enzymes involved in pathway. 4. Expression or deletion of required enzyme in host bacteria. 5. Gene mutation effects on the enzyme properties 6. Assembly of group of genes and their co-expression. Now a day's along with bacteria and yeast, plant cell, animal cells and fungi are also used for metabolic engineering (Kell *et al.*, 2005).

Some of the strategies of metabolic engineering for the achievement of production of required biochemical are discussed below. 1. One of the most commonly used strategies is over expressing of the gene encoding the rate-limiting enzyme of the biosynthetic pathway of the required end product. 2. In this way, we can achieve the overproduction of the desired product by inhibiting or deleting the genes responsible for the competing metabolic reactions which use the same substrate. Through this way the substrate is metabolically channeled particularly towards the desired chemical. 3. There is

possibility of production of the desired biochemical or product in the non-native organism. The genes can be isolated from the native organisms which can produce the required product and these genes can be expressed in another non-native organism (heterologous host organisms). It is mandatory that the required substrate should be available in the non-native organism. Multiple genes representing the group of enzymes of particular pathway can be expressed in the non-native host. Expressing the group of genes encoding the most capable enzymes from diverse organisms is another way to obtain the product which is produced in low quantities or not produced (Patil *et al.*, 2005). Figure 1 explains the strategies for metabolic engineering for the production of a desired chemical.

5. Tools for metabolic engineering

Metabolic engineers use different protein engineering techniques and directed proteomic techniques to get useful information about protein levels in the microorganisms. Metabolic engineers believe that protein engineering and targeted proteomics are very valuable tools that fill the gap to engineer novel metabolic pathways for microorganisms. Synthetic biology had its application in the metabolic engineering for the alteration of microbes for the biorenewable production of biofuels and bioremediation (James, *et al.*, 2016). Through synthetic biology we can design or redesign and construct new biological components such as cells, enzymes, proteins and pathways. The main goal of systems biology is the explaining the cell physiology and function through the integrated use of broad physiological and genomic data. Directed evolution and genetic engineering are the main tools of metabolic engineering which are very much responsible for the its success (Leber and Da Silva, 2014, Meerza *et al.*, 2016). The tools of metabolic engineering are shown in Figure 2.

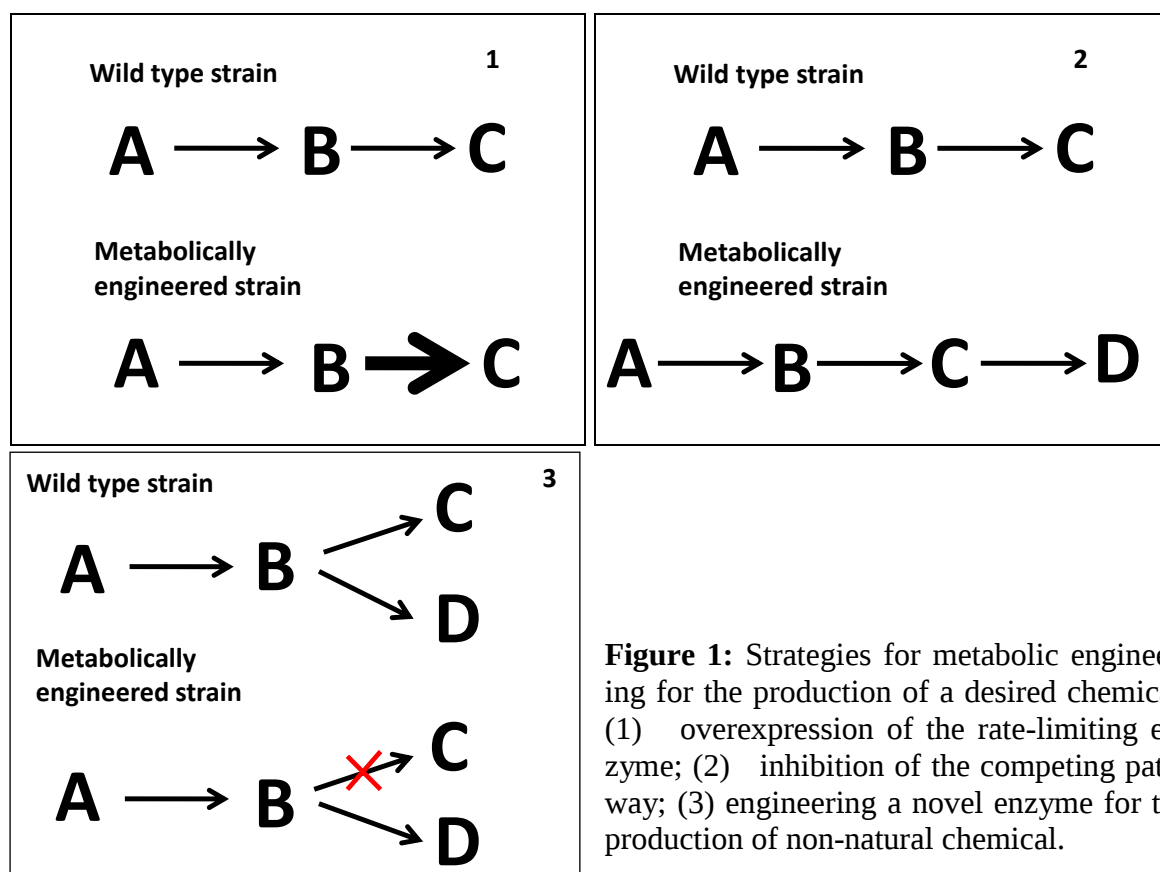


Figure 1: Strategies for metabolic engineering for the production of a desired chemical; (1) overexpression of the rate-limiting enzyme; (2) inhibition of the competing pathway; (3) engineering a novel enzyme for the production of non-natural chemical.

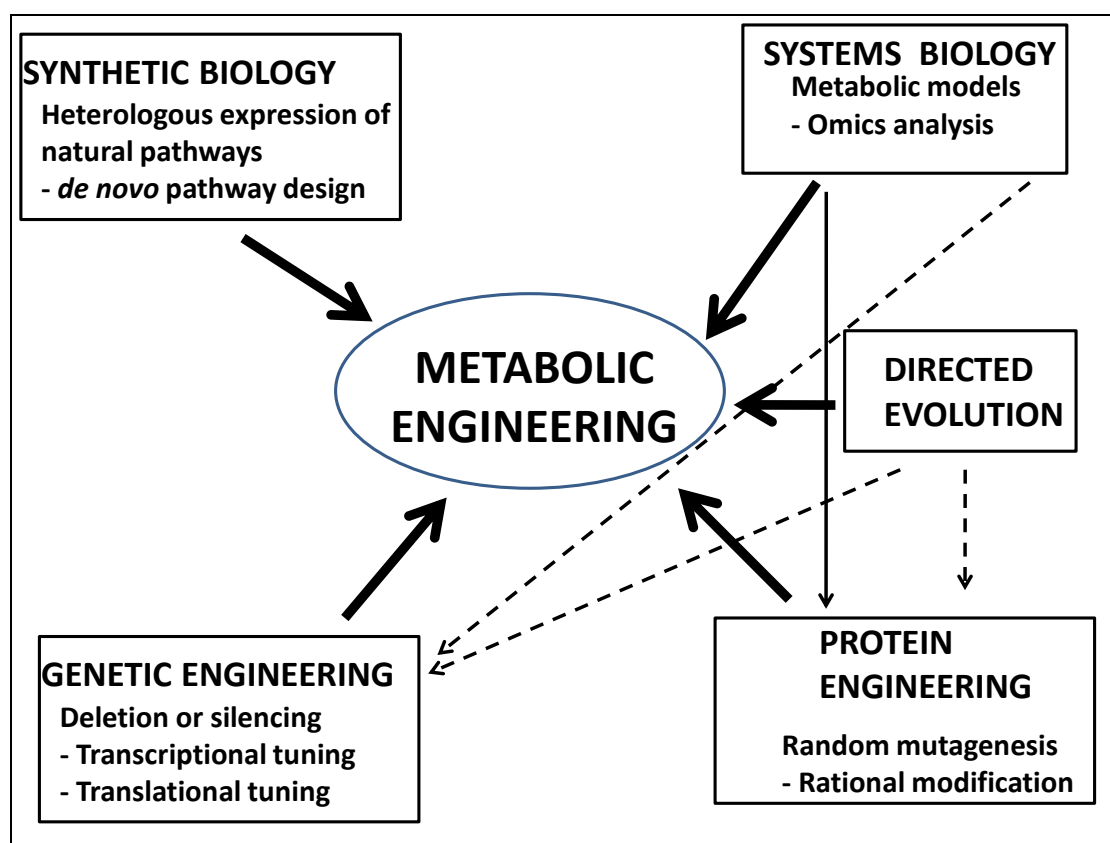


Figure 2: Overview of tools for metabolic engineering.

6. Perspectives

Unquestionably, microbial metabolic engineering is the main tool for production of biofuels and for bioremediation. The scientific and technological progress in metabolic engineering has been estimated to make a good contribution to promote green and sustainable energy by biofuels production. The key advantages of biofuels produced by the metabolically engineered microbes over petroleum based fuels are they are very environmentally friendly, produced from the renewable feedstocks and very low emissions of carbon. From the microbial metabolic engineering standpoint, the discovery and design of novel pathways and enzymes for metabolic engineering of microorganisms coupled with efficient innovative and low cost processing technologies will contribute significantly to the success of production of biofuels and bioremediation through which we can meet the global challenges of environment and climate change. Metabolically engineered microbes could be used as platforms towards green energy and biochemical production, another crucial area of application promises to the human health.

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Biodiesel Production for Sustainability: An Overview

R. Meena Devi, R. Subadevi and M. Sivakumar*

Energy Materials Lab, #120, School of Physics, Alagappa University, Karaikudi-630 004, Tamil Nadu, India; *Correspondence: susiva73@yahoo.co.in; Tel: +91 4565 223304

Abstract: Diminishing petroleum reserves and increasing environmental regulations are the main driving forces to search for renewable fuel. Biodiesel is a renewable substitute fuel for petroleum diesel fuel and produced by transesterification in which oil or fat is allowed to react with a monohydric alcohol in the presence of a catalyst. This paper gives an overview of the work carried out by researchers in the field of biodiesel production from different types of oil. The different methods of biodiesel production techniques and factors affecting biodiesel production are also highlighted.

Keywords: Biodiesel, catalyst, transesterification, vegetable oil, viscosity

1. Introduction

The demand for energy is increasing in the world due to the rapidly growing global population and urbanization. The depletion of world petroleum reserves and increased environmental concerns has stimulated the search for alternative renewable fuels that are capable of fulfilling an increasing energy demand in a sustainable manner (Narasimharao *et al.*, 2007). In recent decades, research concerning and knowledge about the external benefits of renewable raw materials have intensified the efforts for sustainable energy sources.

The various alternative fuel options tried in place of hydrocarbon oils are mainly biogas, producer gas, ethanol, methanol and vegetable oils. Out of all these, biodiesel offers an advantage because of their comparable fuel properties with that of diesel. The emissions produced from biodiesel are cleaner compared to petroleum-based diesel fuel. Biodiesel can be regarded as an alternative diesel fuel.

Biodiesel is the colloquial name for “fatty acid alkyl ester” (FAAE). According to the American Society for Testing and Materials (ASTM), biodiesel

is defined as the monoalkyl esters derivative from lipid feedstocks, such as vegetable oils or animal fats (Avhad and Marchetti, 2016). The dominant biodiesel production process, namely transesterification, typically involves the reaction of an alkyl-alcohol with a long chain ester linkage in the presence of a catalyst to yield mono-alkyl esters (biodiesel) and glycerol (Verma *et al.*, 2016).

Due to the recent increased awareness and development in this area, the objective of this review is to give fundamental insight into the production of biodiesel by different raw materials. Also, this paper, reviews the factors affecting biodiesel production process such as temperature, reaction time, methanol to oil molar ratio, type and amount of catalyst, mixing intensity and fuel properties of biodiesel.

2. Merits and demerits of biodiesel

Some of the advantages of using biodiesel as a replacement for diesel fuel are (Knothe, 2006; Romano *et al.*, 2006):

- Renewable fuel, obtained from vegetable oils or animal fats.
- Low toxicity, in comparison with

diesel fuel.

- Degrades more rapidly than diesel fuel, minimizing the environmental consequences of biofuel spills.
- Lower emissions of contaminants: carbon monoxide, particulate matter, polycyclic aromatic hydrocarbons, aldehydes.
- Lower health risk, due to reduced emissions of carcinogenic substances.
- No sulfur dioxide (SO₂) emissions.
- Higher flash point (100 °C minimum). May be blended with diesel fuel at any proportion; both fuels may be mixed during the fuel supply to vehicles.
- Excellent properties as a lubricant.
- It is the only alternative fuel that can be used in a conventional diesel engine, without modifications.
- Used cooking oils and fat residues from meat processing may be used as raw materials.

There are certain disadvantages of using biodiesel as a replacement for diesel fuel that must be also taken into consideration:

- Slightly higher fuel consumption due to the lower calorific value of biodiesel.
- Slightly higher nitrous oxide (NO_x) emissions than diesel fuel.
- Higher freezing point than diesel fuel. This may be inconvenient in cold climates.
- It is less stable than diesel fuel, and therefore long-term storage (more than six months) of biodiesel is not recommended.
- May degrade plastic and natural rubber gaskets and hoses when used in pure form, in which case replacement with Teflon components is recommended.
- It dissolves the deposits of sediments and other contaminants from diesel fuel in storage tanks and fuel lines, which then are flushed away by the biofuel into the engine, where they can cause problems in the valves and

injection systems. In consequence, the cleaning of tanks prior to filling with biodiesel is recommended. It must be noted that these disadvantages are significantly reduced when biodiesel is used in blends with diesel fuel.

3. History of biodiesel

Dr. Rudolf Diesel actually invented the diesel engine to run on a myriad of fuels including coal dust suspended in water, heavy mineral oil, and, vegetable oil. Dr. Diesel's first engine experiments were catastrophic failures. But by the time he showed his engine at the World Exhibition in Paris in 1900, his engine was running on 100% peanut oil. Dr. Diesel was visionary. In 1911, he stated that "the diesel engine can be fed with vegetable oils and would help considerably in the development of agriculture of the countries which use it". In 1912, Diesel said, "The use of vegetable oils for engine fuels may seem insignificant today. But such oils may become in course of time as important as petroleum and the coal tar products of the present time". No doubt, this statement has come to stay. Since Dr. Diesel's untimely death in 1913, his engine has been modified to run on the polluting petroleum fuel we now know as "diesel." Nevertheless, his ideas on agriculture and his invention provided the foundation for a society fueled with clean, renewable, locally grown fuel. Today throughout the world, countries are returning to using this form of fuel due to its renewable source and reduction in pollution (Owolabi *et al.*, 2012).

4. Oil crops in India

The various oil sources are classified as edible and non-edible. The edible sources like groundnut, peanut etc are primarily used to meet the food requirement. India is not using vegetable oils derived from rapeseed & mustard, soybean or oil palm for the production of

biodiesel. It is because; India is not self-sufficient in edible oils production and depends upon imports of palm oil and other vegetable oils in large quantities to meet the domestic demand. However, utilization of non-edible seed oils extracted from trees and forest sources does not interfere with food security directly if the trees are grown on marginal/waste land that does not compete with food production. Every year around 1.2 million tonnes of tree borne non-edible seed oils are produced in the country (Dwivedi *et al.*, 2011). In India, biodiesel is produced mostly from the non-edible oils extracted from the seeds of plants like *Jatropha*, *Pongamia*, *Mahua*, *Neem* etc. Depending on climate and soil conditions, different nations are looking for different vegetable oils as substitute of diesel fuel for example soybean oil in USA, rapeseed and sunflower oils in Europe, palm oil in south East Asia and coconut oil in Philippines are being considered as substitutes for diesel.

Table 1 summaries the non-edible oil producing plants that can be cultivated for oil production on suitable land and consequently the oil can be used for

biodiesel production (Agarwal, 2007). The non-edible oil seed plant given in the above table has potential to produce oil and subsequent conversion to biodiesel apart from their uses for illumination, burning, soap making, candle making etc. It is estimated that the potential availability of such oils in India is about 2 million tons per year. The most abundant oil sources are *Sal*, *Mahua*, *Neem*, *Pongamia* and *Jatropha* oil. Based on extensive research, *Jatropha* and *Pongamia* have been identified as the potential feed stocks for biodiesel production in India.

The future demand for biodiesel in India is given in Table 2. The above table indicates that by the year 2020–2021, about 24.61 MT of diesel could be saved if B20 blend is utilized. This will ensure sustainable fuel availability with secured environmental conditions. As per the report of the committee on biofuel, the estimated demand of diesel in 2011–2012 was 64.19 MT, requiring 12.84MT of biodiesel and plantation of *Jatropha curcas* over about 13.69 million hectare of land. As per Government of India survey, out of total land area, 124.7 mill-

Table 1: Production of non-edible oils in India

No	Botanical Name	Local Name	Annual Productivity (Tons)
1.	<i>Jatropha curcas</i>	Ratanjyot	45,000
2.	<i>Pongamia pinnata</i>	Karanja	135,000
3.	<i>Schleichera oleosa</i>	Kusum	25,000
4.	<i>Azadirachta indica</i>	Neem	1,00,000
5.	<i>Shorea robusta</i>	Sal	1,80,000
6.	<i>Modhuca indica</i>	Mahua	1,80,000

Table 2: Projections of biodiesel demand and corresponding *Jatropha* area required for meeting the blending targets in India (Area in Mha, Demand in Mt)

Year	Diesel demand	For 5% blending		For 10 % blending		For 20 % blending	
		Biodiesel demand	<i>Jatropha</i> area	Biodiesel demand	<i>Jatropha</i> area	Biodiesel demand	<i>Jatropha</i> area
2011-12	64.19	3.21	3.42	6.42	6.85	12.84	13.69
2016-17	92.15	4.61	4.91	9.21	9.83	18.43	19.66
2020-21	123.06	6.15	6.56	12.31	13.13	24.61	26.25

-ion hectare is classified as waste and degraded land (Dwivedi *et al.*, 2014).

4.1. Typical oil crops useful for biodiesel production

The main characteristics of typical oil crops that have been found useful for biodiesel production are summarized in the following paragraphs.

4.1.1. Castor seed

The castor oil plant grows in tropical climates, with temperatures in the range 20–30°C; it cannot endure frost. It is important to note that once the seeds start germinating, the temperature must not fall below 12 °C. The plant needs a warm and humid period in its vegetative phase and a dry season for ripening and harvesting. It requires plenty of sunlight and adapts well to several varieties of soils. The total rainfall during the growth cycle must be in the range 700–1,400 mm; although it is resistant to drought, the castor oil plant needs at least 5 months of rain during the year. Castor oil is a triglyceride, ricinolenic acid being the main constituent (about 90%). The oil is non-edible and toxic owing to the presence of 1–5% of ricin, a toxic protein that can be removed by cold pressing and filtering. The presence of hydroxyl groups in its molecules makes it unusually polar as compared to other vegetable oils.

4.1.2. Jojoba

Although jojoba can survive extreme drought, it requires irrigation to achieve an economically viable yield. Jojoba needs a warm climate, but a cold spell is necessary for the flowers to mature. Rainfall must be very low during the harvest season (summer). The plant reaches its full productivity 10 years after planting. The oil from jojoba is mainly used in the cosmetics industry; therefore, its market is quickly saturated.

4.1.3. *Jatropha*

Jatropha is a shrub that adapts well to arid environments. *Jatropha*

curcas is the most known variety; it requires little water or additional care; therefore, it is adequate for warm regions with little fertility. Productivity may be reduced by irregular rainfall or strong winds during the flowering season. Yield depends on climate, soil, rainfall and treatment during sowing and harvesting. *Jatropha* plants become productive after 3 or 4 years, and their lifespan is about 50 years. Oil yield depends on the method of extraction; it is 28–32% using presses and up to 52% by solvent extraction. Since the seeds are toxic, *jatropha* oil is nonedible. The toxicity is due to the presence of curcasin (a globulin) and *jatrophic* acid (as toxic as ricin).

4.1.4. *Microalgae*

Microalgae have great potential for biodiesel production, since the oil yield (in liters per hectare) could be one to two orders of magnitude higher than that of other raw materials. Oil content is usually from 20 to 50%, although in some species it can be higher than 70%. However, it is important to note that not all microalgae are adequate for biodiesel production. High levels of CO₂, water, light, nutrients and mineral salts are necessary for the growth of microalgae. Production processes take place in raceway ponds and photobiological reactors.

5. Biodiesel production techniques

There are different processes which can be applied to synthesize biodiesel such as direct use and blending, micro emulsion process, thermal cracking process and the most conventional way is transesterification process (Gashaw *et al.*, 2015).

5.1. Direct use and blending

The direct use of vegetable oils in diesel engine is not favorable and problematic because it has many inherent failings. Even though the vegetable oils have familiar properties as biodiesel fuel, it required some chemical modification

before can be used into the engine. It has only been researched extensively for the past couple of decades, but has been experimented with for almost hundred years. Although some diesel engine can run pure vegetable oils, turbocharged direct injection engine such as trucks are prone to many problems.

5.2. Microemulsion process

A micro emulsion is defined as the colloidal equilibrium dispersion of optically isotropic fluid microstructures with dimensions generally in the range of 1–150 nm formed spontaneously from two normally immiscible liquids and one or more ionic or non-ionic. The problem of the high viscosity of vegetable oils was solved by micro-emulsions with solvents such as methanol, ethanol, and 1-butanol. The components of a biodiesel micro-emulsion include diesel fuel, vegetable oil, alcohol, surfactant and cetane improver in suitable proportions. Alcohols such as methanol and ethanol are used as viscosity lowering additives, higher alcohols are used as surfactants and alkyl nitrates are used as cetane improvers. Microemulsions can improve spray properties by explosive vaporization of the low boiling constituents in the micelles. Micro-emulsion results in reduction in viscosity increase in cetane number and good spray characters in the biodiesel. However, continuous use of microemulsified diesel in engines causes problems like injector needle sticking, carbon deposit formation and incomplete combustion.

5.3. Thermal cracking (pyrolysis)

Pyrolysis is defined as the conversion of one substance into another by means of heat or heating with the aid of a catalyst. Pyrolysis involves heating in absence of air or oxygen and cleavage of chemical bonds to yield small molecules. The pyrolysis of vegetable oil to produce biofuels has been studied and found to produce alkanes, alkenes, alkadienes, aromatics and carboxylic acids in various

proportions. The equipment for thermal cracking and pyrolysis is expensive for modest biodiesel production particularly in developing countries. Furthermore, the removal of oxygen during the thermal processing also removes any environmental benefits of using an oxygenated fuel. Another disadvantage of pyrolysis is the need for separate distillation equipment for separation of the various fractions. Also the product obtained is similar to gasoline containing Sulphur which makes it less ecofriendly. The pyrolyzed material can be vegetable oils, animal fats, natural fatty acids and methyl esters of fatty acids.

5.4. Transesterification

Generally, biodiesel is produced by means of transesterification. Transesterification is the reaction of a lipid with an alcohol to form esters and a byproduct, glycerol. It is, in principle, the action of one alcohol displacing another from an ester, referred to as alcoholysis (cleavage by an alcohol). In Transesterification mechanism, the carbonyl carbon of the starting ester (RCOOR_1) undergoes nucleophilic attack by the incoming alkoxide (R_2O^-) to give a tetrahedral intermediate, which either reverts to the starting material, or proceeds to the transesterified product (RCOOR_2). Transesterification consists of a sequence of three consecutive reversible reactions. The first step is the conversion of triglycerides to diglycerides, followed by the conversion of diglycerides to monoglycerides, and finally monoglycerides into glycerol, yielding one ester molecule from each glyceride at each step. The reaction is represented in equation 1.



Equation 1 Chemistry of transesterification

There are different transesterification processes that can be applied to synthesize biodiesel: (a) base-catalyzed transesterification, (b) acid-catalyzed transesterification, (c) enzyme-catalyzed transesterification, and (d) supercritical alcohol transesterification.

5.4.1. Catalysts: acid catalyst

The use of an acid catalyst is observed to be more effective than alkali catalysts when the concentration of free fatty acids is high. Also the performance of the acid catalyst is not strongly affected by the presence of FFAs in the feedstock. In fact, acid catalysts can simultaneously catalyze both esterification and transesterification. Thus, a great advantage with acid catalysts is that they can directly produce biodiesel from low cost lipid feedstocks, generally associated with high FFA concentrations (low-cost feedstocks, such as used cooking oil and greases, commonly have FFAs levels of >6%)³. However, Homogeneous acid catalyzed reaction is about 4000 times slower than the homogeneous base-catalyzed reaction. Acids used in the catalysis of the transesterification of biodiesels are usually either hydrochloric acid or sulfuric acid. Though these two acids are the most common, any Bronsted acid can also be used in this reaction.

5.4.2. Base catalyst

Transesterification reaction can be catalyzed by both homogeneous (alkalies and acids) and heterogeneous catalysts. The used alkali catalysts are NaOH, CH₃ONa, and KOH for producing biodiesel (Wang *et al.*, 2007). The alkali catalyzed transesterification of vegetable oils proceeds faster than the acid catalyzed. But the use of base catalyzed transesterification is only limited to oil having low water and FFA content. This reaction is the most widely used process for production of biodiesel worldwide. To keep check on the water and FFA content of the oil, they are first pretreated with an

acid catalyzed transesterification process, which converts the FFA to esters (Leung and Guo, 2006).

5.4.3. Enzyme catalysts

Lipase enzymes can also catalyze methanolysis of triglycerides. The most promising results were obtained by using immobilized *Candida Antarctica* lipase (Novozym 435). Shimada *et al.*, (1999), found that Novozym435 was inactivated by shaking it in a mixture containing more than 1.5 M eq. of methanol to oil. Above this concentration, methanol is partially present as small droplets in the oil phase. These droplets are believed to cause enzyme deactivation. Therefore, methanol was added stepwise; after the addition of the third methanol equivalent, conversion to methyl esters was almost complete. The enzyme could be reused 50 times without loss of activity. The occurrence of free fatty acids did not affect the enzyme catalyst. Before the inlet of every reactor, 1 M eq. was added to the feed. Samukawa *et al.* (2000) reported a dramatic increase of the lipase efficiency when it was pretreated by a consecutive incubation in methyl ester and oil prior to reaction. The use of Novozym435 in methanolysis of triglycerides is also reported in supercritical carbon dioxide at 24.1 MPa and 50 °C. High yields (90–95%) of fatty acid methyl esters could be obtained when the reaction was carried out at molar methanol/oil ratios of 25:1.

5.4.4. Supercritical transesterification

Saka and Kusdiana (2001) have developed a catalyst free method for biodiesel fuel production by employing supercritical methanol. The supercritical treatment at 350 °C, 43 MPa, and 240 s with a molar ratio of 42:1 in methanol is the optimum condition for transesterification of rapeseed oil to biodiesel fuel. The great advantage of this method was that free fatty acids present in the oil could be simultaneously esterified in the supercritical solvent. Variables such

as the molar ratio of alcohol to vegetable oil and reaction temperature were investigated during the transesterification within this supercritical media. Increasing the reaction temperature within the supercritical regime resulted in increased ester conversion.

6. Previous work done on production of biodiesel from edible oil

Leung and Guo (2006) compared the transesterification reaction conditions for fresh canola oil and used frying oil. Higher molar ratio (7:1, methanol/used frying oil), higher temperature (60° C) and higher amount of catalyst (1.1 wt% NaOH) was maintained in used frying oil when compared to fresh canola oil where optimal conditions maintained were 315-318 K, 1.0 wt% NaOH and 6:1 methanol/oil molar ratio. However, less reaction time (20 min) was observed for used frying oil when compared to fresh canola oil reaction time (60 min). Ying *et al.* (2011), developed a new method catalyst, benzyl bromide-modified calcium oxide (CaO) for production of biodiesel from rapeseed. The improved catalytic activity was obtained by better fat diffusion to the surface of the benzyl bromide-modified CaO. Further, a 99.2% yield of fatty acid methyl esters in 3h was obtained in comparison to by better fat diffusion to the surface of the benzyl bromide-modified CaO. Wakil *et al.* (2012), chosen Cottonseed oil, Mosna oil and Sesame oil for producing biodiesel.

7. Previous work done on production of biodiesel from non-edible oil

Mohibbe *et al.* (2005), found that FAME of *Jatropha curcas* were most suitable for use as bio- diesel and met the major specification of bio-diesel standards of the European, Germany and USA Standards Organization. Chakrabarti and Ahmad (2008) presented work on extraction of oil from castor bean and converting it into biodiesel. It was

found that reaction mixture containing 65ml of methanol along with 2.4 g of catalyst (KOH) took a good start in half an hour at 30°C. In this reaction, amount of glycerine removed as well as ester content produced was considerably increased with rise in temperature of mixture up to 70°C by extending time period (180-360 minutes). The removal of glycerine increased by two and half times and ester content by four times, respectively. When castor oil was subjected to acid esterification, prior to transesterification (a separate investigation), it was found that ester contents up to 95% could be obtained. Hasan *et al.* (2013) produced biodiesel from neem seeds, its properties were close to diesel. The methodology of esterification process was selected and carried out by 1000 ml raw neem oil, 300ml methanol and sodium hydroxide on mass basis as a catalyst usually kept in oven to form methyl ester, and initially to reach equilibrium condition at temperature 55-66°C. The ester and glycerine were separated by stimulating continuously and allow settling under gravity for 24 h. Thus the separated ester contains 3% to 6% methanol and soap agents. The methanol was removed by vaporization. The biodiesel had some catalyst; it was removed by warm water mix with ester. Kinematic viscosity lay between 1.9 and 6.0 according to the ASTM D6751 specification. It was reported that, 0.95 L biodiesel was produced from 1 L neem oil.

8. Factors affecting biodiesel production

The yield of biodiesel in the process of transesterification is affected by several process parameters which include; reaction time, reaction temperature, catalyst and molar ratio of alcohol and oil and mixing intensity (Gashaw *et al.*, 2015).

8.1. Temperature

Reaction temperature is the important factor that will affect the yield of biodiesel. For example, higher reaction temperature increases the reaction rate and shortened the reaction time due to the reduction in viscosity of oils. However, the increase in reaction temperature beyond the optimal level leads to decrease of biodiesel yield, because higher reaction temperature accelerates the saponification of triglycerides and causes methanol to vaporize resulting in decreased yield. Usually, the transesterification reaction temperature should be below the boiling point of alcohol in order to prevent the alcohol evaporation. The range of optimal reaction temperature may vary from 50°C to 60°C depends upon the oils or fats used. Therefore, the reaction temperature near the boiling point of the alcohol is recommended for faster conversion by various literatures. At room temperature, there is up to 78% conversion after 60 minutes, and this indicated that the methyl esterification of the FFAs could be carried out appreciably at room temperature but might require a longer reaction time.

8.2. Reaction time

The increase in fatty acid esters conversion observed when there is an increase in reaction time. The reaction is slow at the beginning due to mixing and dispersion of alcohol and oil. After that the reaction proceeds very fast. However the maximum ester conversion was achieved within < 90 min. Further increase in reaction time does not increase the yield product i.e. biodiesel/mono alkyl ester. Besides, longer reaction time leads to the reduction of end product (biodiesel) due to the reversible reaction of transesterification resulting in loss of esters as well as soap formation.

8.3. Methanol to oil molar ratio

One of the most important parameters affecting the yield of biodiesel is the molar ratio of alcohol to triglyceride. Stoichiometrically, 3 moles

of alcohol and 1 mole of triglyceride are required for transesterification to yield 3 moles of fatty acid methyl/ethyl esters and 1 mole of glycerol is used. In order to shift the reaction to the right, it is necessary to either use excess alcohol or remove one of the products from the reaction mixture. The second option is usually preferred for the reaction to proceed to completion. The reaction rate is found to be highest when excess methanol is used (Gashaw and Lakachew, 2014). Methanol, ethanol, propanol, butanol and amyl alcohol can be used in the transesterification reaction, amongst these alcohols methanol is applied more frequently as its cost is low and it is physically and chemically advantageous (polar and shortest chain alcohol) over the other alcohols. In contrast, ethanol is also preferred alcohol for using in the transesterification process compared to methanol since it is derived from agricultural products and is renewable and biologically less offensive in the environment.

8.4. Type and amount of catalyst

Biodiesel formation is also affected by the concentration of catalyst. Most commonly used catalyst for biodiesel production is sodium hydroxide (NaOH) or Potassium hydroxide (KOH). The type and amount of catalyst required in the transesterification process usually depend on the quality of the feedstock and method applied for the transesterification process. For a purified feedstock, any type of catalyst could be used for the transesterification process. However, for feedstock with high moisture and free fatty acids contents, homogenous transesterification process is unsuitable due to high possibility of saponification process instead of transesterification process to occur.

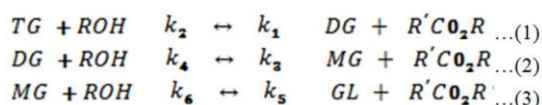
8.5. Mixing intensity

Oils and alcohols are not totally miscible, thus reaction can only occur in the interfacial region between the liquids

and transesterification reaction is a moderately slow process. So, Mixing is very important in the transesterification process, adequate mixing between these two types of feedstock is necessary to promote contact between these two feed stocks, therefore enhance the transesterification reactions to occur (Jagadale and Jugulkar, 2012).

9. Thermo-kinetics of transesterification

The feasibility of a reaction is determined from the thermodynamic parameters. Since both reactants and products are liquids, entropy change will tend to zero, hence equilibrium constant will be low (Owolabi *et al.*, 2012). Kinetics of transesterification reaction has at least 3 main reactions as shown in the equations stated below (Noureddini and Zhu, 1997).



Activation energy for the reverse reaction is higher than that for the forward reaction, which again should confirm the low possibility of reverse reactions. Some of the few kinetics studies that have been performed in recent

times include esterification of free fatty acids in sunflower oil and oleic acid (Berrios *et al.*, 2007 and Kraai *et al.*, 2008). Transesterification kinetics of soybean oil with five different catalysts has also been studied (Singh and Fernando, 2007).

10. Fuel properties of biodiesel

The fuel properties of biodiesel are discussed below (Owolabi *et al.*, 2012; Gopal and Karupparaj, 2015). The limits of ASTM D 6751 standard are listed in Table 3.

10.1. Specific gravity and density

Density is the mass of unit volume of a material at a specific temperature. A more useful unit used by the petroleum industry is specific gravity, which is the ratio of the weight of a given volume of a material to the weight of the same volume of water measured at the same temperature. Specific gravity is used to calculate the mass of oils. Density influences the efficiency of the fuel atomization for airless combustion system. It has some effect on the break-up of fuel injected into the cylinder. In addition, more fuel is injected by mass as the fuel density increases.

Table 3: ASTM D 6751 standard for biodiesel

Parameters	Units	ASTM D 6751 Limits	ASTM D 6751 Methods
Acid number	mg KOH/g	0.50 max	D 664
FFA	%	-	-
Specific gravity	g/cm ³	0.87 - 0.90	D 1250 - 08
Kinematic viscosity at 40°C	cSt	1.9 - 6	D 445
Peroxide value	meq/kg	-	-
Calorific value	MJ/kg	-	D 240 - 02
Sulphated ash	%	0.02	D 874
Water & Sediments	%	0.05	D 2709
Copper corrosion	-	No. 3 max	D 130
Carbon residue	%	0.05	D 4530
Flash point	°C	130 min	D 93

10.2. Lower calorific value

It is a measure of the energy produced when the fuel is burnt completely which also determines the suitability of methyl ester as an alternative to diesel fuel. The calorific value of methyl ester is normally lower than diesel due to oxygen content of methyl ester.

10.3. Iodine value

Iodine value or iodine number is defined as the number of grams of iodine taken up by 100 g of oil or fat. In this case, addition reaction takes place across the double bonds of unsaturated fatty acids present in the fat by the addition of a halogen, such as iodine. Thus, the iodine number gives the indication of the degree of unsaturation of fats.

10.4. Molecular weight

The average molecular weight of the methyl ester is calculated by considering the weight percent of each fatty acid and their corresponding molecular weights.

10.5. Cetane number

It is the measure of the ignition quality of diesel fuel; higher this number the easier it is to start a standard (direct-injection) diesel engine. It denotes the percentage (by volume) of cetane (chemical name Hexadecane) in a combustible mixture (containing cetane and 1-Methylnaphthalene) whose ignition characteristics match those of the diesel fuel being tested.

10.6. Flash point

It is the minimum temperature of the fuel at which the fuel gives flash when it comes to contact with testing flame. It is an important parameter from the safety point of view such as safe for transport, handling, storage purpose and safety of any fuels. This is higher than petrol diesel which has flash point of 70°C. A fuel with high flash point may cause carbon deposits in the combustion chamber.

10.7. Pour point, cloud point

The pour point of a crude oil or product is the lowest temperature at which oil is observed to flow under the conditions of the test. Handling and transporting oils and heavy fuels is difficult at temperatures below their pour points. Often, chemical additives known as pour point depressants are used to improve the flow properties of the fuel. The temperature at which wax crystals begin to form on the surface of the biodiesel is the cloud point.

10.8. Kinematic viscosity

This is the resistance to flow of oil. Ease of starting depends on viscosity. Glycerin contamination may cause biodiesel viscosity to increase. It is the most important fuel features and this factor affects the operation of fuel injection, blending formation and combustion process. The high viscosity interferes with the injection process and leads to insufficient atomization.

11. Concluding remarks

Biodiesel, of the family of biofuel, has been described in this review as a fuel with necessary potentials to replace fossil diesel in future. The trials biodiesel and its blend have undergone is a confirmatory test to all advantages including environmental benefits accrued to it thereby plays a vital role in meeting future fuel requirements. The availability of major feedstock namely oil from biosources and simplicity of the transesterification technology that ensures its conversion to biodiesel are added advantage in terms of the future needs of biodiesel. The use of inedible oil and waste frying/cooking oil has equally assisted in establishing a balance between energy and food security. However, serious efforts have to be intensified on design of large scale bio-refineries for future biodiesel production.

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***In vitro* Cell Bioassays in Pollution Assessment**

Narayanan Kannan^{1,*}, Poorani Krishnan² and Ahmad Zaharin Aris³

¹Postgraduate Research and Innovation and Strategic Development, Taylor's University (Lakeside Campus), No. 1, Jalan Taylor's, 47500, Subang Jaya, Selangor Darul Ehsan, Malaysia; ²Department of Medical Microbiology and parasitology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia; ³Faculty of Environmental studies, Department of Environmental Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia; *Correspondence: Kannan.Narayanan@taylors.edu.my; Tel: +603 5629 5463

Abstract: Most fast developing countries in Asia face severe pollution problems due to industrialization and modernization. Analytical chemical measurements are common in pollution monitoring. However, the ecological (biological) effects of those chemicals are difficult to understand. The latest generation of bioanalytical tools, such as *in vitro* transactivation bioassays, show great promise as water and sediment quality monitoring tools. A battery of *in vitro* cell bioassays has been developed in recent years that are effective, specific, fast, less expensive and easy to handle. One example is, Aryl hydrocarbon Receptor (AhR) mediated CYP1A1 protein expression that has been utilized effectively as a biomarker of pollution stress to marine biota. Hence, AhR binding potential, subsequent induction, genotoxic expression and cytotoxic potential of pollutants are reviewed. The list of *in vitro* cell bioassays reviewed will hopefully be utilized in ecotoxicological studies in developing countries in Asia.

Keywords: Developing countries; ecotoxicology; *in vitro* cell bioassays; monitoring; policy; pollution

1. Introduction

There is a growing concern over persistent organic pollutants (POPs) that are ubiquitous, persistent and toxic (Kannan *et al.* 1988, 1989 a,b,c, 1997, 2016; Kaw and Kannan 2016). Hence, monitoring these chemicals in the environment is important. Conventional monitoring of targeted chemicals relies on analytical methods for measurements in the matrix of interest (i.e. water or sediment). Monitoring can also be based on surveys on the health of individuals and populations of sentinel species (e.g. indigenous fish); or at a larger scale, the integrity of ecosystem health by measuring community composition, diversity and or function. Though these approaches are effective,

they are not very fast, at times, cumbersome and usually expensive, as new chemicals keep entering the aquatic/terrestrial environment. Moreover, this approach does not address unknown chemicals such as photolytic/biological transformation products or complex chemical mixtures that sentinel species of interest are exposed to.

Thus, chemical measurement of priority pollutants alone is not sufficient, as their biological impacts need to be assessed. Toxicity end-points such as survival and mortality of an organism are very useful indicators of biological impacts. However, most of the contaminants occur at low concentrations for any possible toxicological investigation. High throughput cell bioassay techniques have

recently been shown to effectively screen environmental contaminants based on their biological mode of action. In particular, a number of *in vitro* cell bioassays have been adopted to measure the integrated response of bioactive contaminants, such as estrogens, in recycled and surface waters as well as in wastewater effluents (van der Linden *et al.* 2008; Leusch *et al.* 2010; Escher *et al.* 2014; Mehinto *et al.* 2015). These emerging bioanalytical tools can indicate which classes of chemicals are of concern, thus narrowing the field of targeted chemical analysis needed and toxicity endpoints to evaluate (Brack *et al.* 2015; Maruya *et al.* 2016). In general, *in vitro* bioassays are good markers of biological toxicity as they are sensitive, quick, less expensive (Mehinto *et al.* 1993; Qiao *et al.*, 2006). The advantage of effect-based bioassay measurements is that they determine the integrated toxic potency of the complex mixture of micro contaminants in the environment. In addition, bioassays may detect mixture effects of compounds, even when the individual constituents of the mixture are present at concentrations too low to cause an effect or to be detected by chemical analysis (Hamers *et al.*, 2010). The impact of unmonitored contaminants (contaminants of emerging concern) remains largely uncharacterized. Considering these points, one can safely conclude that *in-vitro* bioassays are comprehensive and holistic and can act as early warning signals of environmental degradation.

2. Significance of cell *in vitro* bioassays

In vitro cell bioassays utilizing either wild type cells or genetically engineered eukaryotic cells providing an assessment on the potency of contaminants extracted from environmental matrices. Applications of *in vitro* cell bioassays are highly effective in terms of cost and time. A notable advantage is that through *in vitro* bioassays the detection and assessment of toxicity of a complex mixture of pollu-

tants that exist in environmental extracts is made possible. Moreover, in comparison to the limited identification of pollutants provided through instrumental analysis, *in vitro* techniques provide an assessment of the total biological impact exerted by complex mixtures of environmental contaminants that are mediated through a common mechanism of action. Various bioassays have been developed over the years to evaluate the toxic potency of environmental extracts with reference to a specific target receptor. Bioassays are reliable tools to measure the response of a cell in terms of protein expression and enzyme activity when biological organisms are exposed to environmental pollutants. These responses are reportedly triggered by specific genes that mediate the transcription of the particular target protein. Cell lines developed from mammals and fish utilizing CYP1A1 induction as a biomarker of exposure to Poly aromatic hydrocarbons (PAHs), Polychlorinated biphenyls (PCBs) and other Halogenated aromatic hydrocarbons (HAHs) are effective *in vitro* tools for cumulative impact of sediment extracts (Jung *et al.*, 2012; Schnell *et al.* 2013). Thus, various cell lines developed from mammals (He *et al.*, 2011; Willet *et al.*, 1997 a, b) and fish (Fernandes *et al.*, 2014; Schnell *et al.* 2013; Fent, 2001) inducing CYP1A monooxygenases enzyme belonging to the cytochrome P450 family have been utilized.

3. Aryl hydrocarbon Receptor (AhR) mediated toxic response

The most potent inducer of Aryl hydrocarbon Receptor (AhR) is 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Thus chemicals that elicit response similar to TCDD are generally clustered as dioxin-like chemicals. The ability of these chemicals to cause hepatotoxicity, embryotoxicity, teratogenicity, immunotoxicity, dermal toxicity, lethality, carcinogenesis, and tumor promotion in many different species at low concentrations

have generated much concern (Giesy *et al.*; 2002; Ahlborg *et al.*, 1992; Peterson *et al.*, 1993). These chemicals are largely mediated through AhR dependent mechanism of action in biological system (Poland and Knutson., 1982; Willet *et al.*, 1997, Yoo *et al.*, 2006). Several reviews have been published on the potency of dioxin-like environmental xenobiotics (Gillesby and Zacharewski, 1998; Ankley *et al.*, 1998; van den Berg *et al.*, 1998; Giesy *et al.*, 2002).

AhR is described as a ligand dependent transcription factor located in the cytosol, chaperoned with heat shock proteins (Giesy *et al.*, 2002). Increased toxicity, enhanced gene transcription and enzyme activity are indicators of the binding strength of congeners to the AhR (Giesy *et al.*, 2002; Safe, 1995). The AhR mediated mechanism is initiated once the ligands bind to cytosolic AhR. The receptor ligand complex is then translocated into the nucleus leading to the dissociation of heat shock proteins followed by formation of a dimer by binding to Ah Receptor Nuclear Translocator (ARNT) protein. The heteromeric ligand AhR:ARNT complex then binds to dioxin-responsive element (DRE) a specific DNA sequences. This binding in turn will stimulate transcriptional activation of adjacent responsive genes leading to production of specific protein such as CYP1A1 (Giesy *et al.*, 2002; Denison and Heath-Pagliuso, 1998; Hankinson, 1995; Celander *et al.*, 1996). CYP1A1, belonging to the superfamily of cytochrome P450 plays a significant role in the biotransformation of xenobiotic such as PAHs and PCBs in organisms. Induction of CYP1A has been the most useful biomarker for environmental contamination and had been applied in various pollution monitoring programs (Bucheli and Fent, 1995; Celander *et al.*, 1996).

Biotransformation is a complex process of excretion of hydrophobic substrate by converting them to hydrophilic metabolite through monooxygenation (Czeka, 2000). Bioassays that measure the activation of

AhR signaling pathway through the expression of the protein CYP1A and its enzymatic activity has been developed and constantly improved with modifications to evaluate and applied in environmental assessment.

4. AhR active compounds

AhR ligands are made by hydrophobic compounds such as polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDDs and PCDFs), chlorinated azobenzenes and azoxybenzenes, polychlorinated biphenyls (PCBs), several polycyclic aromatic hydrocarbons (PAHs), polychlorinated naphthalenes (Giesy *et al.*, 2002; Blankenship *et al.*, 2000; Jung *et al.*, 2012) and various halogenated aromatic hydrocarbon (HAHs) (Willet *et al.*, 1997). Relatively weak AhR ligand has been identified for natural and synthetic compounds (Giesy *et al.*, 1998; Denison and Heath-Pagliuso, 1998).

5. Dioxin responsive (DR) CALUX bioassay

The CALUX (chemically activated luciferase expression) is a reporter-gene based cell bioassay that is increasingly applied in screening of dioxin and dioxin like chemicals in environmental matrices and in food materials (Tsutsumi *et al.*, 2003; Cederberg *et al.*, 2002). To perform this assay, recombinant mammalian cell lines that have been stably transfected with one of two different AhR-responsive luciferase reporter gene plasmids that responds to dioxin and related chemicals are utilized (Denison *et al.*, 2004; Garrison *et al.*, 1996). Briefly, these cells contains AhR, that when bound to activating ligands will initiate the transcription of the luciferase genes and the induction of the luciferase activity that will be determined by measuring luminescence. Induction of luciferase activity is directly proportional to the amount and potency of inducing chemical to which the cells are exposed (Han *et al.*,

2004; Denison *et al.*, 2004). However, the results obtained will vary significantly depending on the type of cell lines used. This is due to the species and tissues specific differences and functionality of the AhR as well as the antagonistic and synergistic effects of some compounds that are cell line dependent.

The analysis of the luminescence measured through this assay is converted to CALUX – TEQ toxic equivalents. The luminescence value given by a sample is compared to the dose response curve of a reference standard such as 2,3,7,8-tetrachlorodibenzo-p-dioxin. Briefly, the sample concentration that induce luciferase to 25% of the TCDD-induced maximum luciferase activity is designated as the EC₂₅ TCDD for that sample. The TEQ concentrations were calculated as;

$$TEQ\text{ pg/g} = \frac{TCDD\text{ EC}_{25}\text{ (pg/ml)}}{Extract\text{ EC}_{25}TCDD\text{ (g/ml)}}$$

(1) (Keiter *et al.*, 2008)

H4IIE-luc cells are among the most common cells utilised in luciferase based assay to characterize the induction potency of AhR active compounds such as halogenated aromatic hydrocarbon (HAH) mixtures containing polychlorinated biphenyls (PCBs), dibenzo-p-dioxins (PCDDs) in environmental matrices (Yoo *et al.*, 2006; Giesy *et al.*, 2002; Whyte *et al.*, 2004; Willet *et al.*, 1997 a,b; Schmitz *et al.* 1995). H4IIE-luc cells have been studied and suggested as an alternative bioanalytical tool to the wild-type cells for the detection of AhR agonists in environmental samples (Sanderson *et al.*, 1996).

Progressive studies in this particular assay lead to the development of a third generation (G3) CALUX cell bioassays. In such studies mouse hepatoma (hepa1c1c7) cells transfected with G3 CALUX plasmids with increased numbers of dioxin response elements (DREs) are utilized. This development

provides a highly responsive and sensitive bioassay system for the detection and relative quantitation of very low levels of dioxin-like chemicals in sample extracts (He *et al.*, 2011). This bioassay is an essential assay to characterize AhR active compounds in environmental matrices as it accounts for the total AhR mediated activities in the test samples including non-dioxin-like chemicals such as PAHs and HAHs. Several advantages of CALUX assay are discussed by Windal *et al.* (2005) such as the reliability of CALUX assay to analyzes the overall biological activity of all AhR ligands (agonists and antagonists) present in an extract, in comparison to chemical analyses that only focuses on a selected number of compounds. Besides, for environmental screening CALUX and chemical analysis are complimentary reflecting the magnitude of dioxin like activity induced by other compounds that were undetected in chemical analysis; particularly, when large numbers of environmental samples are to be screened. CALUX ensures a rapid and cost effective analysis for dioxin-like chemicals.

6. EROD Assay

Induction of 7-ethoxyresorufin-O-deethylase (EROD) activity has been applied in numerous environmental toxicology studies as to indicate pollution stress (Fernandes *et al.*, 2014; Kim *et al.*, 2013; Schnell *et al.*, 2013; Huuskonen *et al.*, 2000). EROD activity is based on the deethylation of the synthetic model substrate 7-ethoxyresorufin by CYP1A1, an enzyme that belong to the phase-1 group of biotransformation enzymes. This reaction will produce a fluorescent product resorufin that is quantified fluorometrically and normalized to the total protein content. In biological system, the induction of CYP1A1 is triggered when dioxin-like chemicals such as PAHs and HAHs bind to the AhR. As an immediate response, a binding enhanced gene expression of CYP1A1 protein occurs to detoxify the

system from the xenobiotics. A study conducted on ethoxyresorufin-O-deethylase (EROD) activity of 19 PAHs in the fish hepatoma cell line PLHC-1 has shown that it is a useful tool for the ecotoxicological evaluation of landfill leachates (Fent and Batscher, 2000). Previously, EROD assay was conducted mostly to study the traditional environmental pollutants such as PAHs, PCBs and HAHSs. Recently, EROD assay is being utilized for studying various new kinds of compounds/pollutants such as heterocyclic aromatic compounds containing nitrogen, sulfur or oxygen (NSO-HET) (Hinger *et al.*, 2011). EROD assay is also reported to be used in determining the CYP1A induction potencies of Nitrated polycyclic aromatic hydrocarbons (NPAHs) and N-heterocyclic aromatic hydrocarbons (azaarenes) in fish hepatoma for the first time (Jung *et al.*, 2001). These compounds are commonly found PAH-contaminated environmental samples.

To reflect the induction potency in ecotoxicological evaluation, a concept of induction equivalency factors (IEFs) was developed based on half maximal effect concentration (EC₅₀). EC₅₀ is the concentration of substance that induces 50% of the maximum induction level. IEF values are calculated as follows;

$$IEF = \frac{EC_{50} \text{ (standard reference)}}{EC_{50} \text{ (test compound)}} \quad (2)$$

As for the analysis involving binary mixtures, the EC₅₀ of both single compounds and mixtures were determined and the IEF of the compounds in relation to a reference substance is determined. Induction equivalents (IEQs) for a mixture are then calculated to show the additive interactions of independent compound in a mixture.

$$IEQ_{\text{calculated}} = \sum_{i=1}^n (\text{Concentration of test compound } i \text{ in mixture} \times IEF \text{ of test compound } i) \quad (3)$$

$$IEQ_{\text{Detected}} = \text{Total Concentration of mixture} \times IEF \text{ of mixture} \quad (4)$$

By relating the experimentally detected EC₅₀ value of the mixture to reference compound and multiplying this value by the total concentration of compounds present in the mixture, an IEQ_{det} is determined and compared to the IEQ_{calc} (Jung *et al.*, 2001). The IEQ values are expressed in mg reference substance equivalents (Reference substance equivalents /L).

The 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), the most potent ligand to the aryl hydrocarbon receptor and the strongest inducer of EROD activity in most test systems has been used as a reference substance in many studies (Huuskonen *et al.*, 2000; Celender *et al.*, 1996), however, recent studies suggest that β-naphthoflavone is proved to be the most suitable reference for the routine *in vitro* EROD assay (Heinrich *et al.*, 2014; Fernandes *et al.*, 2014; Schnell *et al.*, 2013).

Many other alternatives have been developed to analyze the data generated through EROD assay. A formula developed by Sprague and Ramsay (1965) are also utilized in which the EROD activity is converted to toxic units (Schnell *et al.*, 2013).

$$TU = \left[\frac{1}{EC_{50}} \right] 100 \quad (5)$$

According to Lapa *et al.* (2002) and Wilke *et al.* (2008) the toxicity is expressed in four categories: highly toxic (TU ≥ 100), very toxic (10 ≤ TU < 100), toxic (1 ≤ TU < 10) and no toxic (TU < 1).

7. Enzyme-linked immunosorbent assay (ELISA)

In the determination of CYP1A enzymatic activity through EROD assay, quantification of CYP1A protein induction level provides important and complementary information about the regula-

tion of CYP1A and mechanism of toxic action. Enzyme-linked immunosorbent assay (ELISA) is a useful technique in immunologically detecting CYP1A protein. ELISA provides complementary information on the induction of CYP1A in the presence of inducing substances. Previously quantification of CYP1A protein content was made possible through the preparation of subcellular fractions involving scrapping of cells from culture flask, followed by sonication and centrifugation and eventually quantification through western blotting (Hahn *et al.*, 1993). This technique is rather time consuming. Hence, a much rapid and sensitive detection of CYP1A protein was developed in which the detection of the amount of CYP1A protein extracted was measured directly in the microwells (Bruschweiler *et al.*, 1995). This technique allows a semiquantitative determination of the amount of immunoreactive CYP1A protein directly in the microwells in which the cells are cultured. The data of absolute absorption values are presented as the percent of maximal induction of CYP1A and EC₅₀ is determined. Studies have utilized ELISA to quantify CYP1A protein to provide a complementary quantification on CYP1A based EROD activity (Herrero and Castel, 1994; Jung *et al.*, 2001).

8. Genotoxicity assay

8.1. Comet assay

Genotoxicity is of great interest because in the case of chronic exposure situation, the possibility of delayed consequences at the population level is high (Devaux *et al.*, 2011). As such, the application of Comet assay provides an early and universal genotoxicity endpoint revealing the primary DNA damage occurred due to the exposure to environmental pollutants (Frenzilli *et al.*, 2009). Genotoxicity assay such as Comet assay is a biomarker assay providing valuable information on the presence of potential carcinogens and mutagens in the envi-

ronmental sample. Comet assay (single cell gel electrophoresis) is a technique of measuring DNA damage in eukaryotic cells or disaggregated tissues when exposed to hazardous agents (Azqueta and Collins, 2013). In this assay the DNA is drawn out towards the anode through electrophoresis, forming a comet-like image that is observed with fluorescence microscopy (Azqueta and Collins, 2013). In general, this assay refers to the relaxation of supercoiled DNA in agarose-embedded nucleoids (the residual bodies remaining after lysis of cells with detergent and high salt), which then allows the DNA to be drawn out towards the anode under electrophoresis, forming comet-like images as seen under fluorescence microscopy. DNA break frequency is indicated through the relative amount of DNA in the comet tail (Azqueta and Collins, 2013). The extent of DNA migration are determined as a percentage of DNA in the tail (% tDNA) using an image analysis system. For statistical analysis, the induction factor (IF) was calculated using the following equation:

$$IF = \frac{\text{mean value of \% tDNA at each concentration}}{\text{mean value of corresponding negative control}}$$

(6) (Šrut *et al.*, 2011)

Concentration dependent induction factor (CDI) index is then calculated by integrating all the important information. This forms the basis for a general comparison of the genotoxic potential of samples in the Comet assay. CDI is calculated according to the following equation:

$$CDI = \sum_{i=1}^n \frac{IF_i}{c_i} \quad (7)$$

IF_i = induction factor of the concentration *i*; *c_i* = concentration *i*; *n* = *n* concentrations (Seitz *et al.*, 2008)

Recent advancement in this particular assay suggests that digestion with lesion-specific enzymes such as Formamidopyri-

midine DNA glycosylase (FPG), provides a greater yield for strand breaks and increases the sensitivity of the assay in general (Azqueta and Collins, 2013). In the case of low level of strand breaks at non-cytotoxic concentrations of genotoxic chemical, the yield of breaks was greatly enhanced after incubation with (FPG) (Azqueta *et al.*, 2013).

This is a sensitive, simple and versatile assay performed to visualize the single strand break in nuclear DNA of single cells (Wilkening *et al.*, 2003). Besides providing the overall level of damage in the cells being analysed, comet assay also provides the data on how the individual cells respond to the xenobiotic. Comet assay has been applied in studies on PLHC-1 fish cell line (Šrut *et al.*, 2011) and characterization of the genotoxicity of sediment extracts from the Baltic and North Sea on EPC (*epithelioma papulosum cyprini*) fish cell line (Kammann *et al.*, 2001, 2004).

8.2. Cytotoxicity assay

To assess the acute toxicity of pollutants on biological organisms, various cytotoxicity tests are usually performed. Among them, tetrazolium salt reduction (MTT) assay is widely used as an endpoint for the cytotoxicity measurement of chemicals/pollutants in monolayer cell cultures (Vakharia *et al.*, 2001; Bruschweiler *et al.*, 1995; Heinrich *et al.*, 2014). The MTT assay detects the reduction of soluble MTT tetrazolium salt to a blue insoluble MTT formazan product by mitochondrial succinate-dependent dehydrogenase (Bruschweiler *et al.*, 1995). The percentage of viability was calculated relative to DMSO treatment control wells from triplicate observations that are designated as 100% viable cells. Only cells with active mitochondria are able to catalyze this reaction. Cellular stress determination is crucial when analyzing complex samples such as environmental samples because enzymatic assays such EROD assay requires an intact metabolism for the *in-vitro* live cell approaches. This in-

tact metabolism ensures the existence of full biotransformation capacity within the cells that is being exposed to the samples. Recent research suggests that the results of *in vitro* EROD assay to be presented by reference to a truly physiological cytotoxicity assay, the 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) test replacing protein normalization which will enable an optimized *in vitro* EROD protocol to a reference compound (Heinrich *et al.*, 2014).

9. Conclusion and recommendations

Pollution is a major setback in developing countries with heavy emphasis on industrialization and modernization. Constant and consistent pollution monitoring is crucial in order to maintain pollution level in control. In pursuit of the effort, extensive environmental toxicology studies are essential because they provide the actual impact situation before the expensive instrumental analysis is conducted. Ecotoxicological studies in Malaysia are extremely rare. Hence, there is a need for immediate exploration and application of these techniques and this will aid the relevant authorities to improve the environmental status monitoring.

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Lipopeptide Biosurfactants from Bioagent, *Bacillus* as a Weapon for Plant Disease Management

Sampath Ramyabharathi, Balaraman Meena*, Lingan Rajendran and Thiruvengadam Raguchander

Department of Plant Pathology, Centre for Plant Protection Studies, Tamil Nadu Agricultural University, Coimbatore – 641-003, Tamil Nadu, India; *Correspondence: meepath@rediffmail.com; Tel: +91 422 6611226

Abstract: Biosurfactants are surface active biological compounds that are produced by broad range of microorganisms. They are ecofriendly with low toxicity, no residual effects with high biodegradable properties and known to suppress the growth of pathogenic fungi. *Bacillus* genus is considered microbial factories for the production of a huge number of biologically active molecules that are inhibitory for plant pathogen growth. In rhizobacteria *Bacillus subtilis*, an average of 4 – 5% of its genome is dedicated to antibiotic synthesis and has the possibility to produce more than two dozen of structurally diverse antimicrobial compounds. Because of the surfactant properties, the antimicrobial peptide compounds or cyclic lipopeptide antibiotics of the surfactin, iturin and fengycin families are well-recognized for their potential applications in biotechnology. Different groups of lipopeptides can give an advantage to the *Bacillus* strains in specific environmental niches. The ability to induce systemic resistance in plants and their use in the spreading of the bacterial cells that leads to rhizosphere colonization could open new fields of applications for their use in phytopharmaceutical products. In this review article, we are highlighting the role and functions of some major biocontrol lipopeptide biosurfactants present in the *Bacillus* species.

Keywords: Biological control; *Bacillus* species; lipopeptide biosurfactants

1. Introduction

Biosurfactants are produced on the microbial cell surface and are capable of lowering surface and interfacial tensions. They are produced extracellularly and thus are potential substitutes for widely used synthetic surfactants. Biosurfactants are widely used in industries, pharmaceutical, agriculture, food, cosmetics, oil production industries and in bioremediation process. Till date a broad range of structurally different biosurfactants have been identified that includes lipopeptides, polysaccharides, proteins, lipoproteins and glycolipids. Lipopeptide biosurfactants are composed of a lipid tail connected to a short linear or cyclic oligopeptide.

The hydrophobic portion of lipopeptides is made up of fatty acids and the hydrophilic portion is composed of peptides or polysaccharides (Georgiou *et al.*, 1992). Hence the presence of hydrophobic and hydrophilic portions within a single molecule, the biosurfactants tend to migrate toward an interface with different degrees of polarity and hydrogen bonding (Desai and Banat, 1997). In Gram-positive *Bacillus subtilis* IAM1213 the production of lipopeptide biosurfactants was first reported (Arima *et al.*, 1968) and the different types of lipopeptide biosurfactants with significant surface activity, antipathogenic (Ramyabharathi and Raguchander, 2014; Ramyabharathi *et al.*, 2016), antinematicidal (Ramyabharathi, 2015;

Sankari Meena *et al.*, 2016) and anti-microbial activity have been reported from other *Bacillus* strains.

2. *Bacillus* lipopeptides

The lipopeptides in *Bacillus* are classified into three families namely Iturin, Surfactin and fengycin family. There are several *Bacillus* strains isolated and reported to produce all the three families of lipopeptide biosurfactants simultaneously (Ramyabharathi and Raguchander, 2014).

2.1. Iturin lipopeptide

Iturins are a member of an anti-fungal lipopeptide group comprises iturin, bacillomycin and mycosubtilin which are cyclic lipopeptides and are linked by beta amino acid residue. Iturin family contains iturins A-E, bacillomycins D, F, and L, and mycosubtilin. Members of this family have powerful antibiotic activity but moderate surfactant activity and enhanced swarming motility (Leclerc *et al.*, 2006). Hence the presence of iturin in *Bacillus* is responsible for disease suppression and growth inhibition of wide number of phytopathogens. *Bacillus subtilis* produces a diversity of antibiotics that are effective against phytopathogenic fungi and bacteria (Phae *et al.*, 1990). Iturin production seems to be constrained to *B. subtilis* and *B. amyloliquefaciens* (Bonmatin *et al.*, 2003). In control of phyllosphere diseases, *Podosphaera fusca* infecting melon leaves the iturins and fengycins lipopeptides produced by *B. subtilis* contributed more in disease suppression. Vater (1986) reported that the iturin is produced, during slow, stationary growth phase. Sandrin *et al.* (1990) reported that iturin A, one member of the iturin group, shows a strong antibiotic activity with a broad antifungal activity, making it an ideal potential biological control agent with the aim of reducing the use of chemical pesticides in agriculture.

The antimicrobial action of *B. subtilis* can be attributed, to a certain

amount, to the production of iturin A (Tsuge *et al.*, 2001). Gene clusters involved in iturin A production have been intensively investigated (Hiraoka *et al.*, 1992, Huang *et al.*, 1993, Kunst *et al.*, 1997, Yao *et al.*, 2003). The iturin A operon spans a region more than 38 kb long and it contains four open reading frames viz., *ituD*, *ituA*, *ituB* and *ituC* (Tsuge *et al.*, 2001). The *ituD* gene encodes a putative malonyl coenzyme A transacylase, whose distraction results in deficiency of iturin A production (Kunst *et al.*, 1997). Ramyabharathi and Raguchander (2014) reported that the *B. subtilis* strain EPCO 16 contains *ItuC*, *ItuD*, *BmyA*, *BacD*, *BacAB* and *FenD* genes involved in the biosynthesis of Iturin, Bacillomycin, Bacilysin and Fengycin, respectively. Iturin and surfactin were detected in culture filtrates from isolate EPCO16 by thin layer chromatography that showed tremendous control over *Fusarium oxysporum* pathogen infecting tomato.

Bacillomycin D which is a member of the Iturin family along with mycosubtilin and iturin A, is made of one β -amino fatty acid and seven α -amino acids exhibits a strong antifungal activity against a broad range of plant pathogenic fungi. Biosynthesis of bacillomycin D is independent of the ribosomal process and the enzymes responsible for bacillomycin D production are complex peptide synthetases. Bacillomycin D and fengycin jointly contributed to the inhibition of conidial germination of *Monilinia fructicola* and fengycin played a major role in suppressing mycelial growth of the fungal pathogen. Luo *et al.*, 2011 performed bioassay of antifungal compound bacillomycin against *Magnaporthe oryzae*, *Rhizoctonia solani* and *Botrytis cinerea*. The hyphae of the pathogenic fungi treated with bacillomycin L showed abnormal growth, and conidia produced enlarged and constricted germ tube. When bacillomycin L used in high concentration there is a possibility for cellular leakage. Bacillomycin D was detected in *B. subtilis* (Moyne *et al.*, 2001; Ramara-

thnam et al., 2007) and *B. amyloliquefaciens* strains. *B. subtilis* AU195 exhibit antifungal and have an amino acid sequence identical to bacillomycin D. There is higher effect of bacillomycin D than of fengycin against various phytopathogenic fungi. (Koumoutsis, 2006; Peypoux et al., 1984).

2.2. Surfactin lipopeptide

The biosurfactant surfactin is an acidic cyclic lipopeptide produced by strains of *B. subtilis*. Among the lipopeptide antibiotics produced by *Bacillus* spp surfactin and Iturin A was most common. The specific surface and membrane active properties of the surfactin help bacteria to form biofilm. It is thought to perform developmental functions rather than disease resistance mechanism in the environment. Surfactin also produces a sturdy membrane-destabilizing action at concentrations even below its critical micellar concentration and induces the arrangement of ion channels in lipid bilayers. Surfactins have been reported to be powerful surfactants due to their outstanding surface activities. Compared with conventional surfactants, surfactin also have significant biological activities, such as antiviral and antibacterial activity. Phae et al., (1990) reported that Iturin A and surfactin producing *B. subtilis* suppressed more than 23 types of plant pathogens *in vitro*.

Surfactin and lichenysin are structurally related lipopeptides produced by *B. subtilis* and *B. licheniformis*. Bonmatin et al., 2003 reported different forms of surfactin with amino acid variation at position 2, 4, and 7. Surfactin bears powerful surfactant properties by declining the surface tension of water from 72 to 27 mN/m at a critical micelle concentration (CMC) of 25–220 mg/L based on its variants and determined conditions. Surfactin is an inhibitor of fibrin clot formation. Surfactins (C12 to C16) were produced simultaneously to increase the antifungal activity of iturin A. Thus exhibits antiviral, anti-tumor, anti-microbial and hemolytic properties. It is required for bio-

film formation of producing cells, swarming motility, and fruiting body formation. However, surfactin also inhibits biofilm formation of other bacteria by interfering with attachment of the cells to surfaces. Surfactin or closely related variants such as lichenysin have been isolated from *B. coagulans*, *B. pumilus* and *B. licheniformis*.

Surfactins are not toxic for fungal pathogens by themselves but they maintain some synergistic effect on the antifungal activity of iturin A. The mode of action of surfactin is act on the phospholipids and is able to form selective ionic pores in lipid bilayers of cytoplasmic membranes. Both surfactin and iturinA are surfactants with a hydrophilic ring of seven amino acids and a long, hydrophobic hydrocarbon tail. Usually the amino acid end stays in the soil and the hydrocarbon tail penetrates inside the pathogen cell membranes. This action creates openings in cell membranes and restricting the growth of many phytopathogens (Ohno et al., 1995; Asaka and Shoda, 1996; Carrillo et al., 2003; Ongena and Jacques, 2008).

IturinA has antibiotic property while surfactin has extremely powerful surface-active property, making its separation much more difficult. Surfactin, which is produced early in the bacterium's growth cycle, has a deep influence on *B. subtilis* colonization of the root surface. Surface motility can be increased in rapid manner and thereby surfactin accelerates the development of multicellular communities which brings colonization of the bacteria referred to as biofilms (Bais et al., 2004; Nagorska et al., 2007; Rudrappa et al., 2008). Biofilm formation in *B. subtilis* added a distinct advantage over many competing organisms in the rhizosphere soil. Poor root colonization by surfactin-deficient *B. subtilis* strains is associated with lack of biocontrol activity (Schippers et al., 1987; Bais et al., 2004).

2.3. Fengycin lipopeptide

Fengycin is a biologically active lipopeptide produced by several *Bacillus subtilis* strains. The third family of lipopeptide biosurfactants includes fengycins A and B, which are also called plipastatins. The structure is composed of a β -hydroxy fatty acid linked to a peptide part comprising 10 amino acids. It is an anti-fungal antibiotic that inhibits filamentous fungi but is ineffective against yeast and bacteria. Fengycin production was identified in *B. cereus* and *B. thuringiensis* in addition to *B. subtilis* and *B. amyloliquefaciens*. It is also capable of inhibiting phospholipase A2 and biofilm formation of several bacteria. These types of lipodecapeptides are produced by various strains of *Bacillus* spp. and exhibit moderate surfactant activities. It shows antifungal activity and more specific for filamentous fungi.

Fengycin produced by *B. subtilis* has antifungal activity against the filamentous fungus. The fungal cell membrane is the primary site for antimicrobial attack by antibiotics fengycin. Fengycins are fewer haemolytic than iturins and surfactins but maintain a strong fungitoxic activity, exclusively against filamentous fungi (Koumoutsis et al 2004; Vanittanakom et al., 1986). Mechanistically, the activity of fengycins is less well known compared with other lipopeptides but they also readily interact with lipid layers and to some extent hold the potential to alter cell membrane structure and permeability in a dose-dependent manner (Deleu et al., 2005). The connection of iturins and fengycins was shown in the antibiosis-based biocontrol activity of *Bacillus* strains against various pathogens and in different plant species. In the case of soil-borne diseases, iturin A produced by *B. subtilis* RB14 is involved in damping-off of tomato caused by *Rhizoctonia solani* (Asaka and Shoda, 1996). *Bacillus subtilis* S499 efficiently produces lipopeptides from the three families, and notably produces a wide variety of fengycins (Jacques et al., 1999). Mutant analyses in *B. subtilis* subsp. *amyloliquefaciens* strain

FZB42 have indicated that fengycin and bacillomycin D act synergistically to inhibit the growth of *Fusarium oxysporum* under *in vitro* condition (Koumoutsis et al., 2004).

3. Biocontrol potential of lipopeptide biosurfactants

Antibiotic production by *B. subtilis* strains plays a major role in suppression of plant diseases (Kinsella et al., 2009). *B. subtilis* EPCO16 has lipopeptide genes viz., *ItuC* gene, *ItuD* gene (Iturin); *BmyA* gene (Bacillomycin A), *BacD* gene (Bacillomycin D), *BacAB* gene (Bacilysin) and *FenD* gene (Fengycin). The presence of lipopeptide antibiotics in *B. subtilis* EPCO16 inhibited the mycelia growth (46.04%) of *F. oxysporum* f. sp. *lycopersici* (Fol) under *in vitro* (Ramyabharathi and Raguchander, 2014). *B. subtilis* strain Bbv 57 is positive for Iturin (*ItuD* gene), Surfactin (*srfA* gene; *sfp* gene), Bacilysin (*bacAB* gene; *bacD* gene), Bacillomycin D (*bamD* gene), Fengycin (*fenB* gene), Ericin (*eriB* gene), Mycosubtilin (*mycC* gene) and Subtilin (*spaB* gene) lipopeptides (Ramyabharathi and Raguchander 2014a). The inhibition of *Fusarium oxysporum* f. sp. *gerberae* might be due to the production of antimicrobial metabolites which are toxic to the pathogen. HPLC analysis for *B. subtilis* Bbv 57 showed 91.69 $\mu\text{g}/\mu\text{l}$ of surfactin with the retention time of 2.304 min and 0.453 $\mu\text{g}/\mu\text{l}$ of Iturin with the retention time of 8.739 min at 205nm whereas the standard iturin and surfactin at 205nm recorded retention time of 8.5 min and 2.5 min respectively.

Crude lipopeptides extracted from the culture supernatant of *B. subtilis* strain, Bbv 57 treatment revealed least egg hatching of 7 juveniles / egg mass with highest juvenile mortality of 87 per cent of *M. incognita* with the 25 per cent concentration of the antibiotic after 72 h of exposure of nematodes in it. It also inhibited the mycelial growth of *F. oxysporum* (28.20 %) at 10 micro litre con-

centrations. Presence of these diverse genes plays a crucial role in biological control of root knot nematode and *Fusarium* in gerbera both in poly house and *in vitro* through synergistic action of antimicrobial peptide genes (Ramyabharathi, 2015). Among lipopeptides iturin have strong broad spectrum antifungal and hemolytic activity. *B. subtilis* isolate ME488 showed positive reaction for PCR detection of antimicrobial peptide gene viz., *ituC*, *tuD*, *bacA*, *bacD*, *mrsA* and *mrsM* (Chung et al. 2008). Ramarathnam et al. (2007) detected lipopeptides antibiotics genes bacillomycin and fengycin using specific primers in *Bacillus* spp.

Several strains of *Bacillus*, has AMP biosynthetic genes *bmyB*, *fenD*, *ituC*, *srfAA*, and *srfAB* responsible for the suppression of plant pathogens (Gonzalez et al., 2010). Presence of antimicrobial peptide (AMP) biosynthetic genes *srfA* (surfactin), *bacA* (bacylisin), *fenD* (fengycin), *bmyB* (bacylloomicin), *spas* (subtilin), and *ituC* (iturin) in 184 isolates of *Bacillus* spp (Mora et al., 2011). Cadena et al., 2008 reported that *B. amylo-liquefaciens* strain FZB42 produced lipopeptides, surfactins, bacillomycin D, and fengycins, which are secondary metabolites with mainly antifungal activity, also decreased gall formation, egg mass count and juvenile counts of *M. incognita* extracted from roots of tomatoes. Koumoutsi et al. (2004) reported that mutant analyses in *B. subtilis* sub sp. *amyloliquefaciens* strain FZB42 with fengycin and bacillomycinD act synergistically and inhibited the growth of *F. oxysporum* *in vitro*. Cellular leakage was also observed when bacillomycin L was used at higher concentration.

4. Concluding remarks

Bacillus strains do have the capability to produce a variety of lipopeptides with outstanding surface active properties, disease reduction and biological actions. Among the three lipopeptides, iturin and fengycin family separately exhib-

ited antifungal, antimicrobial and antinematic activity, whereas surfactin retained the antifungal effect of iturin A as a synergistic factor. The lipopeptides are less toxic and helps in disease reduction with control of phytopathogens and pathogenic nematodes than agrochemicals. *B. subtilis* seems to be a good bio-control agent and a flourishing antagonist with lipopeptide antibiotic production. Further research is needed to know the stability of lipopeptide antibiotics subject to field conditions. The biosurfactant lipopeptides are used in food industry, chemical industry, clinics, cosmetics and used for cleaning oil spills by bioremediation approach. These lipopeptides may be useful in various industries and agriculture as biosurfactants and biopesticides in plant protection, respectively. However, further research is needed to explore the full potential of lipopeptide biosurfactants.

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Biotechnology as a Tool for Conservation and Sustainable Utilization of Plant and Seaweed Genetic Resources of Tropical Bay Islands, India

Pooja Bohra^{1*}, Ajit Arun Waman² and Anuraj Anirudhan³

¹Division of Horticulture and Forestry, ICAR- Central Island Agricultural Research Institute, Port Blair- 744105, Andaman and Nicobar Islands, India; ²Division of Horticulture and Forestry, ICAR- Central Island Agricultural Research Institute, Port Blair- 744105, Andaman and Nicobar Islands, India; ³Division of Fisheries Sciences, ICAR- Central Island Agricultural Research Institute, Port Blair- 744105, Andaman and Nicobar Islands, India; *Correspondence: poojabohra24@gmail.com; Tel.: +91-3192-250436

Abstract: Andaman and Nicobar Islands are known to harbor large diversity of plant and seaweed species, a number of them being endemic. Micropropagation could be used as an effective tool for large scale multiplication of economically important plants and seaweeds. The technique could also help in multiplying threatened species to conserve them. *In vitro* production of pharmaceutical macromolecules could be a viable option for avoiding destructive harvesting of plant species. Somaclonal variation, *in vitro* mutagenesis and transgenic could be useful in some cases. Molecular markers could help in assessment of genetic diversity, DNA barcoding, marker assisted selection etc. The article highlights the importance and relevance of various biotechnological tools in the management of biodiversity of the fragile ecosystem of Andaman and Nicobar Islands. Various research activities undertaken to conserve species of these islands are also highlighted.

Keywords: Andaman and Nicobar Islands; Bay of Bengal; biodiversity; endemism; sustainable development

1. Introduction

Natural resources including flora and fauna have been the major associates of humankind since evolution. We are largely dependent on these resources for our existence and leading a normal day to day life. However, the increasing pressures of manmade and natural disasters have jeopardized these resources in such a way that every year the conservation status of a large number of species is pushed further in the *red* list. On the other hand, our dependence on a few species for meeting most of our requirements has worsened the situation by eliminating the so called non-useful types, which could be carrying potent genes for mitigating

the stresses posed by climate change. The processes of industrialization, commercial synthetic farming, pollution, deforestation, urbanization etc. are the direct or indirect consequences of population explosion, which have largely contributed in misbalancing the resource utilization in a sustainable way. Considering the sensitivity of these issues, concerted efforts are required to protect our valuable resources so that they are available to the future generations too.

The tropical rainforests are known to harbor wide array of unique floral diversity and a number of mega biodiversity hotspots are located in these regions. The Andaman and Nicobar Islands in the Bay of Bengal (a Union Territory of the India)

are strategically placed between two such biodiversity hotspots viz. Arakan Yoma ranges of Myanmar and the Sumatra. This has resulted in unique confluence of flora of both these regions in ANI (Pandey and Diwakar, 2008). The islands are characterized by lush green forests occupying about 81.8% of the total geographical area. There are more than 2,314 species of flowering plants reported from these islands so far (Murugan *et al.*, 2016) and the number may still increase considering the larger unexplored areas. Furthermore, these islands are known to harbor a large number of endemic species in a relatively smaller geographical area of about 8,249 km². So far, about 300 species of endemic plants have been reported from ANI (Murugan *et al.*, 2016). Majority of the diversity is still unexplored and considerable scope exists for utilizing these species for the betterment of humankind. Since, horticulture has been the major source of livelihood and nutritional security for the island dwellers (Singh *et al.*, 2016), the present article focuses on the management of genetic resources of horticultural crops including their wild relatives.

Similarly, seaweeds are important component of the diversity of these islands. The macro algae mainly belonging to Chlorophyta, Phaeophyta and Rhodophyta are found attached to the substratum in benthic zone. They are non-flowering plants with true roots, stem and leaves, and are known to contribute substantially to the primary production in the marine environment. Seaweeds have been used for centuries as food either in raw or processed form in many of the South East Asian countries and the trend is picking up in the western countries as well. Seaweeds are the only known natural sources of hydrocolloids viz. agar, algin and carrageenan. These multipurpose products find application in industrial, pharmaceutical and medicinal fields. Besides, seaweeds are used as animal feed and biofertilizers in crop production. The current research on seaweeds is centering on bio-

actives which are of great interest in the medical field. Andaman and Nicobar Islands, with 1/3rd of India's coastal line, supports good diversity of seaweeds and so far about 206 species including commercially important agarophytes and alginophytes have been reported from here.

Interestingly, different parts of these islands are inhabited by six native tribes since centuries. Two Mongoloid tribes, Shompen and Nicobarese, reside in the Nicobar groups of islands, while the tribes of Negrito origin i.e. Jarawa, Great Andamanese, Onge and Sentinelese, are residing in the Andaman islands. These tribes differ in most of their cultures and habits. Some of the local species are presently being used by the native tribes for food, medicine, fodder, fuel and other purposes. Similarly, the settler population migrated from different parts of mainland India are utilizing these plants for variety of purposes. Underutilized fruits, indigenous leafy vegetables and tuber crops have immensely contributed in the livelihood and nutritional security of the island dwellers. Further, a large number of wild relatives of cultivated crop plants have been reported to occur in these islands. This diversity needs to be assessed and utilized sustainably to strengthen our resource base, while striking the fine balance between development and ecological soundness (Waman and Bohra, 2016).

Biotechnology could be an effective tool for achieving this target through the application of techniques namely micropropagation, *in vitro* production of secondary metabolites, *in vitro* mutation, *in vitro* conservation, marker assisted selection, genetic diversity assessment, development of trait specific markers *etc.* (Waman *et al.*, 2015; Waman and Bohra, 2016). Present chapter concerned exploring the possibility of utilizing various biotechnological tools for management of biodiversity of the tropical Bay Islands of India.

2. Relevance of biotechnological approaches and tools for island ecosystem

2.1. Micropropagation and *in vitro* conservation

Andaman and Nicobar Islands are home to many endemic species belonging to different botanical families, which are of potential economic/ ecological significance and require timely attention for their conservation. A number of species belonging to rare, endangered and threatened (RET) category are also found distributed in these islands. Some of these species have problems in natural regeneration owing to the damage caused by birds/animals, poor seed viability, anthropogenic pressure etc. For example, *Myristica andamanica* is a vulnerable wild nutmeg species endemic to the islands and micropropagation could help to multiply it in large number. Similarly, natural populations of an underutilized fruit species – blood fruit (*Haematocarpus validus*) are dwindling (Bohra et al., 2016a) and micropropagation could help in saving the species from extinction from the region. Experiments are in progress to standardize micropropagation protocol for this species.

Secondly, banana is a major crop of the islands covering more than half of the area under fruit crops cultivation. However, the islands are largely dependent on the planting material supplies from mainland India. This has probably resulted in inadvertent introduction of dreadful banana bunchy top virus in the pristine islands. Developing protocols for *in vitro* multiplication of locally suitable varieties would help in production of their quality planting material. The importance of tissue culture technology for the island farmers has been emphasized earlier (Bohra et al., 2016b). Considering this, experiments have been initiated at authors' institute for optimization of protocols for locally popular banana varieties of the islands. Other commercializable crops of the islands include variety of orchids and ornamental plants, which need further attention. Use of low cost options including concurrent ex vitro rooting cum

hardening has been emphasized in *low price-high value* crops, especially medicinal plants (Waman and Bohra, 2016).

In vitro conservation is a technique, wherein plant tissues are cultured *in vitro* under sub-optimal growth conditions in order to reduce the frequency of sub-culturing. The technique has proven to be very efficient for short to medium term storage of a number of species. Considering vulnerability of the islands to natural disasters, the endemic species could be conserved under *in vitro* conditions and copies of the same could be maintained at other laboratories in mainland India. Some horticulturally important endemic species of islands have been listed in Table 1.

2.2. *In vitro* production of secondary metabolites

A large number of species are valued for their medicinal properties. However, the yield of bioactive molecules is very low in most of the cases. At times, complete plants are destroyed for obtaining the desired active ingredients. Such practice of destructive harvesting has been a cause of concern as it tends to threaten the natural populations to a great extent (Waman and Bohra, 2013). Biotechnological tools could help in large scale quality production of secondary metabolites under *in vitro* conditions. Induction of callus and extraction of active ingredients from them has been suggested as an important alternative for obtaining the desired molecules without disturbing the wild populations (Waman et al., 2015).

2.3. Creation of variability

There are a few species e.g. mangosteen (*Garcinia mangostana*), which are economically important for the islands but have narrow genetic base. For improvement of such crops, creation of variability is possible through the induction of somaclonal variations in tissue culture or using the technique of *in vitro* mutation

Table 1: List of selected endemic species of horticultural importance reported from ANI

Family	Species
Anacardiaceae	<i>Mangifera andamanica</i> , <i>M. nicobarica</i> , <i>Semecarpus kurzii</i>
Apocyanaceae	<i>Carissa andamanensis</i>
Arecaceae	<i>Phoenix andamanensis</i>
Clusiaceae	<i>Garcinia andamanica</i> , <i>G. cadelliana</i> , <i>G. calycina</i> , <i>G. dhanikhariensis</i> , <i>G. kingii</i> , <i>G. kurzii</i> , <i>G. microstigma</i>
Dilleniaceae	<i>Dillenia andamanica</i>
Dioscoreaceae	<i>Dioscorea vexans</i>
Musaceae	<i>Musa bulbisiana</i> var. <i>andamanica</i> , <i>M. indandamanensis</i> , <i>M. sabuana</i> , <i>M. paramjitiana</i>
Myristicaceae	<i>Myristica andamanica</i> , <i>Knema andamanica</i>
Myrtaceae	<i>Syzygium andamanicum</i> , <i>S. manii</i>
Pandanaceae	<i>Pandanus lerum</i> var. <i>lerum</i>
Tiliaceae	<i>Grewia indandamanica</i>
Orchidaceae	<i>Vanilla andamanica</i> , <i>Eulophia nicobarica</i>
Zingiberaceae	<i>Kaempferia siphonantha</i>

breeding. Being cornerstone of breeding activity, variability created will also be useful in development of island suitable varieties.

2.4. Estimation of genetic diversity

The ANI is considered as a centre of origin or diversity for a number of species e.g. wild populations of *Piper betle* are present in these islands. Both inter and intra specific diversity occurs for a number of species of ecological and economic importance (Singh *et al.*, 2016). This diversity needs to be tapped in such a way that commercially viable types are identified for the benefit of island farmers. Selection of such elite types needs systematic characterization. Molecular characterization is one of the most reliable methods of estimating such diversity. Further, available diversity could also be compared with their counterparts occurring in mainland India or other parts of the world. Through this information, the studied population could be categorized in a way to pave the way for future breeding programmes. A few attempts were initiated at the authors' Institute or other organizations in country in this direction, which have been summarized in Table 2. The similar technique could also be employed for diversity estimation and further studies in non-traditional and underutilized

species, which play pivotal role in the lives of island dwellers. The generated information from such studies could be useful for selection of parents for carrying out conventional crop improvement programmes.

2.5. Evolutionary studies

From time to time, a number of new species have been reported by various research workers in the ANI, however, absence of requisite scientific information about their genetic relationship with their existing commercial counterparts could delay the process of their commercial utilization. Molecular phylogenetic studies could help in this regard. For example the presence of Indo-Myanmarese as well as Indonesian forms of *Erianthus arundinaceus* (Retz.) Jeswiet, a wild relative of sugarcane (*Saccharum officinarum*) was confirmed through the use of molecular markers (Nair and Mary, 2006). They concluded that collections from North Andaman were more similar to Indo-Myanmarese form, while that from Nicobar were of Indonesian form. This report supports the fact that ANI harbor confluence of flora of two different regions. Similarly, genetic diversity between the collections of *Musa balbisiana* from mainland India and the islands suggested that ANI is one of

Table 2: Selected examples of application of molecular markers for diversity assessment in various horticultural crops of ANI

Species	Molecular marker used	Salient findings	Reference
<i>Morinda citrifolia</i>	RAPD, ISSR	Distinct clustering of collections from ANI islands	Singh et al., 2012
<i>Morus laevigata</i>	RAPD, ISSR	Significant genetic divergence between collection from mainland India and Andaman Is.	Naik et al., 2015
<i>Carica papaya</i>	ISSR	Geographical clustering of collections from various islands	Sudha et al., 2013
<i>Bouea oppositifolia</i> , <i>Mangifera andamanica</i>	SSR	Genetic similarity of 43% between both the species of Anacardiaceae family	Damodaran et al., 2013
<i>Cocos nucifera</i>	RAPD	Two distinct clusters based on morphological and nut parameters	Sankaran et al., 2012
	SSR	High genetic diversity among the collections, separate clustering of tall and dwarf types	Rajesh et al., 2008
<i>Syzygium cuminii</i>	RAPD, ISSR	Genotype collected from Car Nicobar was distinct amongst 21 island genotypes and 2 mainland genotypes	Ahmad et al., 2012
<i>Colocasia esculenta</i>	RAPD, ISSR	Island collections were distinctly different from 3 released varieties used as reference genotypes	Singh et al., 2012
<i>Mangifera indica</i>	SSR	Separate clustering of monoembryonic, polyembryonic and wild mango species	Damodaran et al., 2012
Orchid species	RAPD	Distinct clustering of green orchid species	Singh and Srivastava, 2010
<i>Costus speciosus</i>	RAPD	Intra-specific variations to the extent of 35%	Mandal et al., 2007

the centres of diversity of the species (Uma et al., 2005). This new piece of information would go a long way in devising conservation strategy of *Musa* spp. from ANI.

2.6. Development of trait linked markers

As previously mentioned, a number of perennial dioecious species are known to occur in the islands. Important being *Myristica andamanica*, *Knema andamanica*, *Horsfieldia glabra*, *Piper betle*, *Carica papaya*, *Garcinia* spp., *Momordica* spp. etc. Development of sex linked markers would be a boon for the conservation and utilization of these species as the desired plants could be identi-

fied during early stages of development. In case of medicinal plants, identification of markers linked to the presence of their active ingredients would be very useful. Flowering behavior related markers in mango have been identified, which could be of practical utility (Damodaran et al., 2006).

3. Applications of biotechnological interventions in seaweeds

In the wake of increasing demand for seaweeds for various applications, collections from the natural habitats are not sufficient to meet the requirement. Artificial culture of commercially exploited

seaweeds still remains the major means of raw material supply to the industries. However, on farm culture of seaweeds using vegetative fragments generally results in reduced growth rate and productivity over time. In order to overcome these problems and to develop strains with improved growth and yield parameters, biotechnological techniques such as micropropagation, transgenics and molecular markers have been tried in seaweed breeding and genetic studies. Micropropagation is a tool to produce large number of seeding material from explants of seaweed possessing desirable traits. Among the three cellular organization types in seaweeds, most of the work on micropropagation has been reported on parenchymatous and pseudo-parenchymatous types (Aguirre-Lipperheide *et al.*, 1995). Although Gibor attempted to cultivate seaweeds axenically in as early as 1950, the first successful attempt is considered to be made during 1978 by Chen and Taylor in *Chondrus crispus* (Yokoya and Valentin, 2011). Seaweed micropropagation protocols have been developed in similar lines with that of higher plants and so far micropropagation protocols have been developed in about 85 species (Reddy *et al.*, 2008). Considering the diversity present and diversified applications of seaweeds, the technique is considered to

be still in nascent stage. *In vitro* derived calli of economically important seaweeds have been commonly used for maintenance and clonal propagation of seed stock for mariculture (Dawes and Koch, 1991; Reddy *et al.*, 2003; Rajakrishna *et al.*, 2004; Reddy *et al.*, 2008). Studies suggested that growth and quality of carrageenan obtained from tissue cultured *Kappaphycus alvarezii* were superior when compared with conventional vegetative fragments (Rajakrishna *et al.*, 2007). Non-availability of standardized protocols for obtaining viable axenic cultures from wild, lack of knowledge about the role of culture incubation conditions, media supplements, explanting season etc. on callus induction are the major limiting factors in the development of seaweed micropropagation. Table 3 represents list of selected seaweed species reported from ANI in which micropropagation has been attempted elsewhere.

Molecular marker assisted breeding based on quantitative trait loci (QTLs) offers several advantages over traditional phenotypic based breeding. Development of markers linked with genes of desirable traits could increase accuracy and efficiency of the breeding process. Several molecular markers such as Random Amplified Polymorphic DNA (RAPD), Inter Simple Sequence Repeat (ISSR), Se-

Table 3: List of seaweed species reported from ANI in which cell and tissue culture has been accomplished elsewhere

	Seaweed	Reference
Chlorophyta	<i>Boergesenia forbesii</i>	Enomoto and Hirose, 1972
	<i>Bryopsis plumosa</i>	Tatewaki and Nagata, 1970
	<i>E. intestinalis</i>	Polne-Fuller and Gibor, 1987; Russig and Cosson, 2001
Rhodophyta	<i>Gracilaria corticata</i>	Subbaraju <i>et al.</i> , 1981; Rajakrishna <i>et al.</i> , 2007
	<i>G. verrucosa</i>	Gusevet <i>et al.</i> , 1987; Kaczyna and Megnet, 1993
	<i>Gelidiella acerosa</i>	Rajakrishna <i>et al.</i> , 2004
Phaeophyta	<i>Grateloupia filipina</i>	Huang and Fujita, 1997; Baweja and Sahoo, 2009
	<i>Hypnea musciformis</i>	Rajakrishna <i>et al.</i> , 2007
	<i>Sargassum tenerrium</i>	Rajakrishna <i>et al.</i> , 2007
	<i>Turbinaria conoides</i>	Rajakrishna <i>et al.</i> , 2007

quence Characterized Amplified Region (SCAR), Amplified Fragment Length Polymorphism (AFLP), Sequence Tagged Site (STS), Microsatellites etc. have been developed in seaweeds (Lin *et al.*, 2012). Molecular markers have also been used for DNA barcoding, which could be used for assessing genetic diversity and taxonomic identification of seaweeds species, which lack reliable morphological characters for identification. For this purpose, RAPD, Restriction Fragment Length Polymorphism (RFLP), AFLP, Microsatellites, Single Nucleotide Polymorphism (SNP) etc. have been used (Liu and Cordes, 2004). Further, molecular markers could also be used for identification of invasive seaweed species, which could pose threats to the pristine biodiversity of these Islands. Transgenic technology has been mainly focused on genetic engineering of economically important seaweeds (Lin *et al.*, 2012) like *Gracilaria*, *Kappaphycus*, *Porphyra* and *Ulva*. As with any other transgenic technology, the safety issues associated with culture of genetically modified seaweeds should be of prime consideration. Transgenic technology has been attempted in Chlorophyta (Huang *et al.*, 1996), Rhodophyta (Wang *et al.*, 2010) and Phaeophyta (Zhang *et al.*, 2008). Production of valuable products in an indoor cultivation system with proper biosafety from transgenic kelp sporophyte has been successfully demonstrated (Qin *et al.*, 2005).

The general perception that compounds and chemicals produced from natural resources are safe and pose less risk to health has brought seaweeds in the limelight for extracting environmental friendly natural compounds and chemicals. The development of species-specific *in vitro* cell and tissue culture technology is essential for use of other biotechnological tools for genetic improvement and production of high value compounds from seaweeds. With plethora of problems faced in seaweed cultivation, the intervention of biotechnological tools is essential for overall growth of this sector.

4. Perspectives

The tropical islands in the Bay of Bengal are known to harbor considerable diversity of flowering plants as well as seaweed species. Most of these species are ecologically important, while a large number of species could be utilized for their commercial potential. The diversified applications of biotechnology could be helpful in carrying out the major activities related to management of this unique diversity including conservation, regeneration, characterization, utilization etc. Though some efforts have been initiated in this direction, there is vast scope for employing biotechnological tools for sustainable development of such fragile ecosystem in near future.

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Plantibodies for Global Health: Challenges and Perspectives

Prasad Minakshi^{1*}, Basanti Brar¹, Manimegalai Jyothi¹, Ikbal¹, Koushlesh Ranjan¹, Upendra Pradeep Lambe¹ and Gaya Prasad²

¹Department of Animal Biotechnology, LLR University of Veterinary and Animal Sciences, Hisar 125004, Haryana, India; ²Sardar Vallabhbhai Patel University of Agriculture and Technology, Meerut 250110, Uttar Pradesh, India; *Correspondence: minakshi.abt@gmail.com; Tel: 09992923330

Abstract: Antibodies are the important part of adaptive immune system. Plants do not naturally make the antibodies; but, they can be produced in plants by introducing antibody-coding genes from humans and animals. Plant derived antibodies are called as plantibodies and known to work in the same way as mammalian antibodies. The plantibodies bioproduction offers several advantages over the production of antibodies using mammals. Plants are more economic than all other forms of creating antibodies and the technology for obtaining and maintaining them is already present. Plantibodies are safer in use because, plants reduce the chance of coming in contact with pathogens. Plantibodies can be made at an affordable cost using plants as the genetic engineering methods are well established for agricultural crops such as tobacco, tomato, potato, soyabean, alfalfa, rice, and wheat. Plantibodies production is cost effective and safe. This review highlights the methods of production and purification of plantibodies as well as the various types of pharmaceutical antibodies produced in transgenic plants.

Keywords: Antibody production; human and animal health; plants

1. Introduction

Plantibody is an antibody that is produced by plants that have been genetically engineered with animal DNA. These plant produced antibodies, namely plantibodies were first demonstrated by Hiatt *et al.* (1989) and Duering *et al.* (1990). They demonstrated that plants can express and assemble functionally active antibodies. Plants are used in this technology as antibody factories, to produce large amount of clinically viable proteins by using the endomembrane and secretory systems of plants and later it will be derived from those plant tissue (Jain *et al.*, 2011). For more than a decade, various kinds of antibody formats have been produced and studied in different species

of plants and today it became the front-runners among plant-derived pharmaceutical proteins. Plants offer many advantages and potential benefits for the production of recombinant proteins in terms of cost, safety and scalability (Stoger *et al.*, 2014). For large-scale needs, production of recombinant protein using transgenic plants as bioreactors is more economical than alternative systems such as cell culture based antibody production. The main anticipated advantage is cost saving, low cost biomass production using agriculture in a short time without any specialized equipment or expensive media. Moreover, scale up process can be achieved quickly and inexpensively by cultivating more land (Stoger *et al.*, 2014). Other important advantage is the

ability of plant cells to correctly fold and assemble antibody fragments, single chain peptides and also full-length multimeric proteins and heterologous proteins such as antibodies accumulate in large amount to plant cells. Protein synthesis, secretion and folding as well as posttranslational modifications like signal peptide cleavage, di-sulphide bond formation and the initial stages of glycosylation are very similar in plants and animals. There is very low risk of product contamination by mammalian viruses, bacterial toxins, blood-borne pathogens and oncogenes (Jain *et al.*, 2011). Use of these plantibodies avoids the ethical issue of producing transgenic animals, elimination of purification requirement when the plant containing recombinant protein is edible and production of disease resistant plants by raising antibodies in them. The plantibodies bioproduction process offers several advantages over the conventional method of antibody production in mammalian

cells with the cost of plantibody production in plants are substantially lesser (Oluwayelu *et al.*, 2016). Different types of plantibodies are produced from plants (Figure 1).

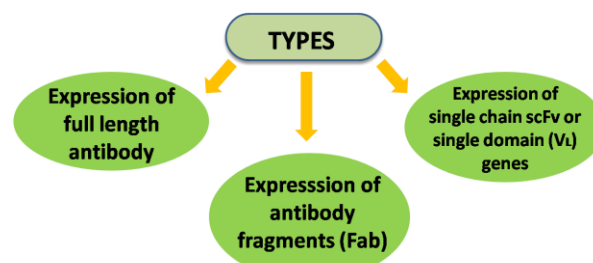


Figure 1: Different types of plantibodies are produced in plants.

2. Plants used as expression host for plantibody production

A range of different plant systems have been developed for antibody production (Table 1). The choice of expression

Table 1: Pharmaceutical antibodies produced in transgenic plants

Antigen	Plant	Antibody form	Application	Reference
Human chorionic Gonadotrophin	Tobacco	scFv, diabody, chimeric, IgG1	Diagnostic/contraceptive	Kathuria <i>et al.</i> , 2002
Glycophorin	Barley, potato, tobacco	ScFv-fusion	Diagnostic (HIV)	Schunmann <i>et al.</i> , 2002
Streptococcus surface antigen SAI/II	Tobacco	SigA/G (CaroRx)	Therapeutic (topical)	Ma <i>et al.</i> , 2003
Sperm	Corn	IgG	Contraceptive (topical)	Cone and Whaley, 2002
Rhesus D	Arabidopsis	IgG	Diagnostic	Bouquin <i>et al.</i> , 2002
Human IgG	Alfalfa	IgG	Diagnostic	Khoudi <i>et al.</i> , 1999
Rabies	Tobacco	IgG	Therapeutic	Ko <i>et al.</i> , 2003
Herpes simplex virus	Soyabean, rice	IgG	Therapeutic (topical)	Zeitlin, 1998
CD40	Tobacco cell culture	ScFv-immunotoxin fusion	Therapeutic	Francisco <i>et al.</i> , 1997
Herpes simplex virus	Algae chlamydomonas chloroplast	One-chain antibody	Therapeutic	Mayfield <i>et al.</i> , 2003

Table 1: Continued...

Antigen	Plant	Antibody form	Application	Reference
Colon cancer antigen	Tobacco	IgG	Therapeutic /Diagnostic	Verch <i>et al.</i> , 1998
Carcinoembryonic antigen (CEA)	Tobacco, rice, wheat, pea, tomato	scFv, diabody, chimeric, IgG1	Therapeutic/Diagnostic	Stoger <i>et al.</i> , 2002
Herpes simplex virus	Corn	sIgA	Therapeutic	Hood <i>et al.</i> , 2002
Non-Hodgkins lymphoma idiotypes	Tobacco Virus vector	scFv	Personalized vaccines	McCormick <i>et al.</i> , 2003
Clostridium difficile	Corn	IgG	Therapeutic (oral)	www.epicyte.com
Hepatitis B virus	Lettuce	IgG	Vaccine	Kapusta <i>et al.</i> , 1999
New castle disease virus	Corn	Surface glycoprotein F	Vaccine	Guerrero-Andrade <i>et al.</i> , 2006
Cholera	Tomato	Cholera toxin B subunit (<i>ctb</i> gene)	Oral vaccine	Jiang <i>et al.</i> , 2007
Enterovirus	Tomato	Serum IgG VP1	Oral vaccine	Chen <i>et al.</i> , 2006
Porcine reproductive and respiratory syndrome virus	Banana	IgG and IgA	Oral immunization	Chan <i>et al.</i> , 2013

system depends upon many factors such as suitability for scale-up, storage and downstream processes. Other considerations attributed to host choice are anticipated production scale, the value and use of the product, geographical area of production, proximity of processing facility, biosafety, intellectual property right and economical aspects (Twyman *et al.*, 2003). Several works have shown that tobacco, potatoes, soya beans, corn, alfalfa and similar kind of crops are the alternative ways for production of recombinant proteins (Hiatt *et al.*, 1989; Mason and Amtzen, 1995). Figure 2 showed that many benefits of plants used for plantibody production.

2.1. Tobacco

Among leafy crops tobacco have the greatest biomass yield per hectare and allow rapid scale up because they can

crop several times in a year (Fischer *et al.*, 2003). Tobacco grows quickly and has been shown to produce comparatively large quantity of antibodies. Additionally, tobacco is a non-food/non-feed crop if grown separately; there is less chance of cross-contamination food chain by pharmaceuticals (Schillberg *et al.*, 2002). Chloroplast engineering in tobacco, an alternative to nuclear transgenics, transplastomic plants are produced by introducing DNA into the chloroplast genome instead of nuclear genome, and this will be achieved by particle bombardment (Daniell *et al.*, 2002). Chloroplast transformation is more advantageous, includes high transgene copy number, absence of position effects and transgene silencing. Combination of these properties leads to extraordinary levels of expression, exceeding 25 percent of the total soluble protein (Tregoning *et al.*, 2003). Other

benefit of chloroplast engineering includes the ability to express several genes as operons and the accumulation of recombinant protein in the chloroplast, which reduces the toxicity of host plant. Biologically active and structurally accurate Human growth hormone and serum albumin produced at high levels in tobacco chloroplasts (Staub *et al.*, 2000; Fernandez *et al.*, 2003). Recently tetanus toxin fragment (Tregoning *et al.*, 2003) and cholera toxin B subunit (Daniell *et al.*, 2001) has been expressed in tobacco chloroplast and was shown that tetanus toxin fragment induce protective levels of anti-tetanus antibodies and cholera B subunit shows that plastids can fold and assemble oligomeric proteins perfectly. Fischer *et al.* (1999) reported that recombinant proteins can also be produced from tobacco cell culture and several recombinant proteins including antibody derivatives were derived from a suspension cell line of tobacco strain BY-2. The expression of classical swine fever virus E2 protein was expressed in tobacco chloroplast elicited protective immune response in mice

which was immunized with leaf extracts conferred specific antibodies (Shao *et al.*, 2008).

2.2. Alfalfa and other legumes

Another leafy crop used to produce recombinant antibodies are alfalfa and soya bean. Alfalfa is a perennial crop can propagated easily and also have good biomass yield. The Biotechnology Company Medicago selected it as a platform technology. The strong advantage of this crop is its tendency to synthesize homogenous N-glycans, which improves the consistency of recombinant proteins batch to batch (Bardor *et al.*, 2003). One of the potential advantages of alfalfa is that recombinant antibodies produced as a single glycoform rather than heterogeneous collection of different glycoforms that is found in other plant systems. Pea, a grain legume also a useful production crop, reason that of its high protein content of seed. Although at present only low yield is possible with this species (Perrin *et al.*, 2000).



Figure 2: Benefits of using plants for plantibodies production.

2.3. Cereals

The disadvantage of tobacco in recombinant protein production is its instability. The leafy tissue needs preservation such as freezing and drying for transportation (Ma *et al.*, 2003). Cereals and legumes produce less biomass when compared to leafy crops but the accumulation of recombinant antibodies in seeds allows long-term storage in ambient temperature because the desiccated environment of the mature seeds reduces the exposure of stored proteins to non-enzymatic hydrolysis and protease degradation. It has been shown that antibodies expressed in rice seeds remains stable in room temperature for years with no detectable loss of activity (Stoger *et al.*, 2000). Seeds of cereals lack phenolic substances, which are present in tobacco leaves, so it increases the efficiency of downstream process (Ma *et al.*, 2003). Several crops have been investigated for antibody production includes, rice, wheat, barley, maize, legume pea and soya bean. Since the said seed crops are used as food, so the downstream processing steps may benefit from the food processing facilities. Maize is now the main commercial crop used for the production of two technical proteins Avidin and β -glucuronidase by a commercial molecular farming venture called Prodigene, and Maize also reflects the advantageous factors such as high biomass yield, ease of scale-up, ease of transformation and *in vitro* manipulation (Hood *et al.*, 1997; Witcher *et al.*, 1998). Maize is also being used for the production of recombinant antibodies, technical/pharmaceutical enzymes such as laccase, trypsin, and aprolinin (Hood *et al.*, 2002a and 2002b). The expression of antibodies in other cereal crops has been explored and experiments have been carried out in wheat and rice (Stoger *et al.*, 2000). The antibody level of $150 \mu\text{g g}^{-1}$ was expressed in transgenic barley was one of the most encouraging result have far come from the expression of a diagnostic scFv fusion protein called SimpliRED (Schunmann *et al.*, 2002).

2.4. Fruits and vegetables

The benefit of fruits, vegetables, and other leafy crops is that they can be consumed raw or partially processed, which make them particularly suitable for the production and expression of recombinant antibodies for passive oral immunotherapy. Storage organs of plants such as tubers combine this advantage with a prolonged shelf-life when compared to leafy crops. Potato have been used widely for the production of plant-derived vaccines and been administered to humans in most of the clinical trials so far. Arts-eanko *et al.*, 1995 first demonstrated the potential of potato tubers for antibody production and recently this crop has been investigated as a bulk-production system for antibodies (Wilde *et al.*, 2002). Potatoes were also used for the production of diagnostic antibody-fusion proteins (Schunmann *et al.*, 2002) and human milk proteins (Chong and Langridge, 2000). Tomatoes have outstanding properties for pharmaceutical protein production, such as high biomass yield and advantage of contained growth in greenhouse, by considering these potentials; tomatoes were used to produce the first plant-derived rabies vaccine (Garvey *et al.*, 1995; stoger *et al.*, 2000). Lettuce was used as a production host to produce edible recombinant vaccine against Hepatitis B virus. Kapusta *et al.*, (1999) shown that mice fed with transgenic lupin tissue were developed significant levels of Hepatitis B virus specific antibodies and also human volunteers who fed with transgenic lettuce plants expressing HBV surface antigen developed specific serum IgG response to plant produced protein. Transgenic tomato based developed edible vaccine expressed cholera toxin B against cholera in the ripening tomato fruit under the control of tomato fruit-specific E8 promoter using *Agrobacterium*-mediated transformation (Jiang *et al.*, 2007). The immunogenicity of the CTB protein expressed in tomato fruit was evaluated through determination of the serum and mucosal anti-CTB antibody levels in experimental mice. A study

conducted by Chen *et al* (2006), proved that VP1 protein of enterovirus in transgenic tomato plant provide both cellular and humoral immunity in orally immunized mice, those fed with tomato fruit expressing VP1 protein. Additionally, serum from mice fed with transgenic tomato could neutralize the EV71 infection to rhabdomyosarcoma cells, indicating that tomato fruit expressing VP1 was successful in orally immunizing mice. Pigs were immunized with recombinant GP5 protein expressed in transgenic Banana leaves using *Agrobacterium*-mediated transformation with ORF5 gene of porcine reproductive and respiratory syndrome virus envelope glycoprotein GP5. Pigs immunized orally with GP5 protein showed a gradual dependent increase in the elicitation of serum and saliva anti-PRRSV IgG and IgA was observed and significantly lower viraemia and tissue viral load were recorded when compared to the pigs which are fed with untransformed banana leaves (Chan *et al.*, 2013).

3. Methods for plantibody production

Various techniques have been developed to exploit plants as bioreactors for the production of plantibodies. Some of the techniques are described below:

3.1. Conventional method

Once the desired DNA from the transformed host cell is isolated and purified, it can be injected into the embryo of a maturing plant, which we want to use for plantibodies production. After injecting the desired gene, followed by propagation of plant in open field allow large scale production of plantibodies. Plant tissue culture is most economic and time saving method for antibody production from plants. In this method, plant cells in differentiated states are grown in bioreactors with foreign proteins harvested either from biomass or culture liquid with less contaminant (Moffat, 1989).

3.2. In vitro cell and tissue cultures

This is an economically important method for producing plantibodies where in plant cells in differentiated stages are grown under controlled conditions having desired genes/ proteins and are harvested either in the form of biomass and culture liquid or combination of both. This method offers large amounts of recombinant proteins production in a shorter time (Doran, 1999). This method offers many advantages over conventional method of plantibodies production by overcoming the problem of extracting and purifying proteins but this method has not used for edible vaccines production. In this method no sexual reproduction is needed, so transgenic stability is increased because of absence of crossing over, segregation and recombination and provides more chances in plantibodies production (Ferrante and David, 2001).

3.3. Breeding and sexual crossing

An experiment on tobacco plant was established for its breeding and sexual crossing as a method for the production of plantibodies. In this experiment, transformation was used to introduce kappa chains of either light or heavy regions into tobacco plants. Same way was done with gamma heavy chains. Upon crossing one plant with kappa-chains and another with gamma-chains, an antibody was produced that expressed both chains (Hiatt *et al.*, 1989; Whitelam *et al.*, 1994). This method provides an easy way to produce plantibodies without the need for double fertilization (Ferrante and David, 2001).

3.4. Transgenic seeds

Above mentioned methods have certain limitations. Further restrictions are found when plants used as storage system because plants cannot store antibodies for a longer time. This is due to certain proteases degrade the protein piece by piece. So, some researchers suggest that, use of transgenic seeds in place of green leafy plants, because seeds can store antibodies for an extended period without degradation. Seeds contain low level of proteases

that allows proteins to be stored for longer period when compared to green plants (Larrick *et al.*, 1998; Fieldler and cornad, 1995). So, seeds can be used as bioreactors and used as natural storage organs (Ferrante and David, 2001).

3.5. Targeting and compartmentalization

Antibodies can be targeted to some compartments by tagging with a small peptide sequence and this allows antibodies to be protected from proteases that present in the cytoplasm (Kusnadi *et al.*, 1997). Compartmentalization to be easily isolated organelles and makes easy purification procedure (Kusnadi *et al.*, 1997). Targeting, however, has to be specifically controlled and this involves proper cleavage of targeted sequences.

4. Purification techniques

The main reason for raising antibodies in plants is its easy purification and low downstream processing. Easy purification of plantibodies makes biopharmaceutical production more economic (Arntzen, 1998). Transgenic seeds assure excellent storage properties and thus added flexibility in processing management and batch production. Separation of plantibody in seeds is less complicated because of limited range of endogenous proteins (Kusandi *et al.*, 1997). Absence of human pathogens in plants eliminates expensive validation of virus removal steps during purification. But the probability of presence of a diverse burden on plants grown outdoors in non-sterile conditions is high. So the process for elimination or minimization of contamination with endotoxin and mycotoxins will be necessary in all commercial process to purify antibodies (Gegenheimer, 1990). Phenolics can interact with proteins in ways that can irreversibly alter the properties of proteins but most of the phenolics released during extraction are small in size, water soluble, and removable by ultrafiltration steps. Main techniques used for the purification of plantibodies are

filtration, immunofluorescence, chromatography, diafiltration, polymer fusion and protein A-sepharose chromatography. Some other techniques such as RIA (Radioimmunoassay), northern blot technique, ELISA (Enzyme linked immune sorbent assay), western blot analysis and immunofluorescence southern blot analysis have been used of evaluation of plantibody.

5. Applications of plantibodies

5.1. Bioreactors

Antibodies produced in plants have applications such as production of vaccine antigens, protein for clinical diagnosis, pharmaceutical and industrial proteins, carbohydrates, vitamins, minerals, biopolymer and food (Sharma and Sharma, 2009). These applications were proved in basic agronomy research (Jaeger *et al.*, 2000). In recent years, many plant systems has been developed in order to using plants as bioreactors for the production of recombinant antibodies for many purposes (Stoger *et al.*, 2002). Using plants as a bioreactor, or as a factories or using them as antibody replacement for microorganisms like bacteria to produce human antibodies to communicate with human health mainly due to two reasons.

(i) When compared to prokaryotes, plants are better for the production of antibodies due to large scale production efficiency and low cost of production.

(ii) Process of post-translational modification such as glycosylation that they are the kind of post-translational modifications of proteins, in plants can be done more carefully than bacteria (Ma and Hein, 1995).

5.2. Therapeutic applications

CaroRx, the first plant derived antibody created from tobacco (Fischer *et al.*, 2006), is a Sig A secretory antibody. It is a clinically advanced anti *Streptococcus mutans* secretory immunoglobulin, a plantibody that binds specifically to the bacterium, thus protecting humans from

dental carries (Larrick *et al.*, 2001). CaroRx is intended for regular topical preventive administration by both dental hygienists and patients allowing a thorough cleaning and intervention for any existing decay. Plantibodies have been investigated for inflammatory disease and to induce tolerance (Jain *et al.*, 2011). A humanised antibody, another plantibody with human medical application was expressed in soya bean against herpes simplex virus (Zietlin *et al.*, 1998). Anti-tumour antibodies against Burkitt's lymphoma was expressed in rice and wheat (Ghasempour *et al.*, 2014). Antibodies engineered to bind to *Bacillus anthracis* was extracted from transgenic strains of tobacco and tested in mice in a study conducted by Hull *et al* in 2005. The result of this study showed that the antibodies were effective in fighting *B. anthracis* strain and bodes well for the future in any anthrax epidemic will be a cheap and effective prevention against the disease.

Treatment or cure for rabies through plantibodies has been investigated by Ko *et al.* 2003. A plantibody based rabies vaccine produced in tobacco was experimentally administered in hamsters to check whether it could effectively target rabies. The plantibody proved to be safe and economically feasible alternative method compared to the current antibody production in animal systems. Another study, tobacco-derived plantibodies were experimentally administered in mice against the Lewis Y antigen found on tumour cells in mice and also in lung, breast, ovarian and colorectal cancer. According to Brodzick *et al* (2006), the plantibodies showed a definite positive effect on the cancer-stricken mice by preventing tumour formation in them (Figure 3).

5.2.1. Immunization

One of the most interesting applications of this technology is production of edible vaccines or oral vaccines. Produc-

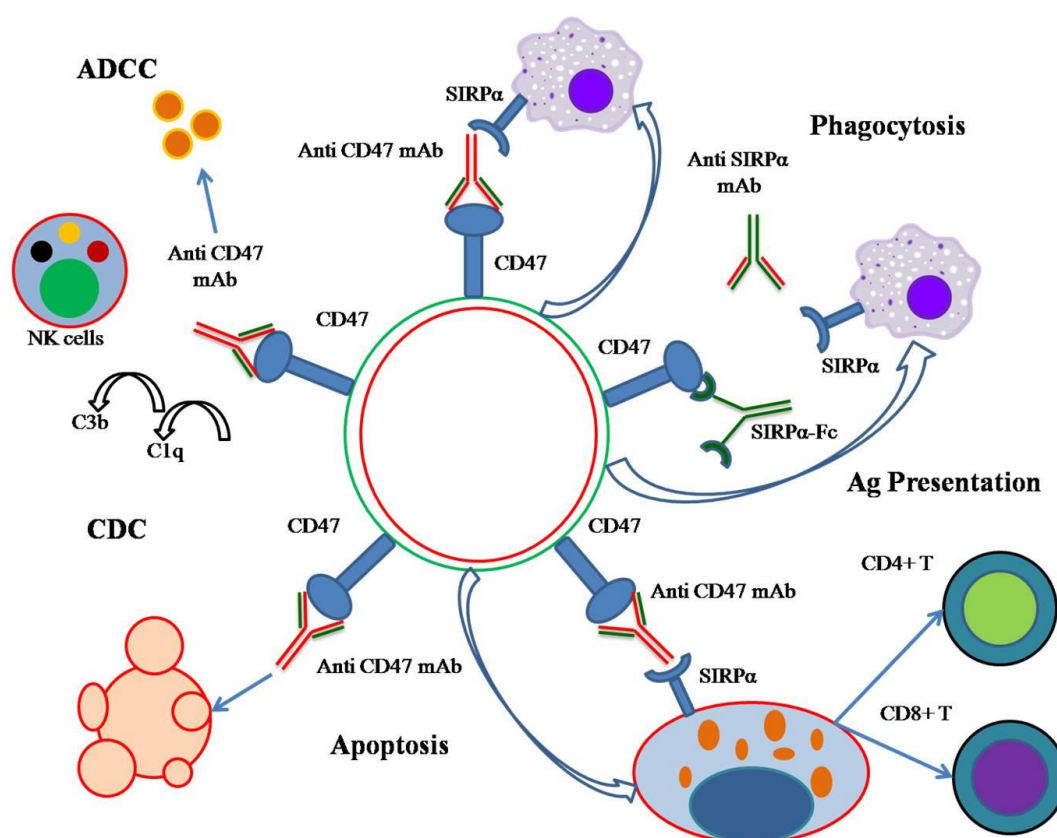


Figure 3: Use of plantibodies for cancer treatment.

Potential proteins such as cytokines, hormones, enzymes, epidermal growth factors, interferons, human protein C, and pharmaceutical foodstuff are produced, which are considered for oral immunization (Mason and Amtzen, 1995). Transgenic plants that express antigens might be used as an inexpensive oral vaccine production and delivery system, so immunization might be possible through consumption of an edible vaccine to provide immunization. Due to all these reasons transgenic plants are considered as better alternative for oral vaccines. This offers convenient immunization strategies for implementing universal vaccination programmes throughout the world (Tacket et al., 1998). In human host the pathogens usually attack mucosal sites in the respiratory tract, gastro-intestinal tract or genital tract. Stimulation of immune responses in these sites through mucosal vaccines to protect against illness is desirable and this can be achieved by applying vaccine to the mucosal surface directly, inducing systemic and cellular immune responses as well as local immune responses at the initial site of interaction between the pathogen and host directly (Kusnadi et al., 1997). Oral vaccines must be protected during passage through the hostile environment of the stomach and intestine to the sites of immune stimulation.

5.2.2. Immunomodulation

Immunomodulation is a technique that allows interference with cellular metabolism, signal transduction or pathogen infectivity by the ectopic expression of gene encoding antibodies or antibody fragments (Jaeger et al., 2000). Applications that are relaying on modulating antigen levels in vivo are dependent on expression and accumulation in specific sub cellular compartments and specific tissues. Development of crop resistance and passive immunization of plants by expression of pathogen-specific antibodies reduces infection and symptoms caused by viruses and mollicutes, and significant

progress has been made towards engineering resistance against insects (Schillberg et al., 2001; Jaeger et al., 2000). Immunomodulation is a dynamic tool for altering the function of an antigen *in vivo*. When an artificial abscisic acid sink was created by the production of an anti-abscisic acid specific scFv in the endoplasmic reticulum of potato and tobacco plants, both physiological and morphological changes were noticed (Conrad and Manteuffel, 2001). Moreover, agro-filtration of tobacco was used to produce a diabody against carcinoembryonic antigen (Vaquero et al., 2002). In addition, plantibodies may also prove useful as feed additives or for phytoremediation in human health care (Mason and Arntzen, 1995).

6. Pathogen resistance in plants

Antibody mediated pathogen resistance in plants is a novel strategy for generating transgenic plants resistant to pathogens have been developed in many cases for therapeutic applications, for Immunomodulation. Peschen et al., (2004) demonstrated antibody-mediated resistance against fungal pathogens and protecting plants against fungal diseases. Schillberg et al (2000), targeted anti-TMV antibodies to the plasma membrane in vivo (in planta) results in involvement of transgenic plants resistant to TMV infection. A study conducted by Boonrod et al (2004), paved the way for engineering broad-range virus resistance by expression of scFv antibodies in vivo that are specific and highly conserved motifs in viral replicase or polymerases. Boonrad and his colleagues demonstrated that transgenic tobacco plants expressing scFv antibodies against a conserved domain in plant viral RNA dependent RNA polymerase either in the cytosol or endoplasmic reticulum, showed high levels of resistance to four plant viruses from different genera. Malembic-Maher et al. (2005) studied the scFv 2A10 protein expression in engineered tobacco plants and for their

resistance to stobular disease. It was observed that tobacco plants producing secreted scFvs shown a short delay in symptom appearance, reduction to pathogen susceptibility and phyloplasma multiplication. Isolated antipectinase scFv antibodies directed against extracellular proteins from *Rhizoctonia solani* secluded from a phage display library. Soluble scFv antibodies shown to inhibit polygalacturonase in the culture supernatants of a range of fungal pathogens such as ascomycetes, basidiomycetes and oomycetes. This soluble antibody also inhibited maceration in potatoes (Manatunga *et al.*, 2005). First time antibody mediated fungal resistance was demonstrated by Wu *et al* in wheat and cereal grains. This fungal resistance in transgenic plants is mediated by generating specific antibodies against *Fusarium graminearum*, a predominant fungal species infecting wheat and small cereal grains in china (Wu *et al.*, 2005).

7. Treatment of Ebola patients

Recently, antibodies against Ebola virus have been explored in plants. A high yielding geminivirus-based expression system in the tobacco plant, *Nicotia-*

na benthamiana, was used for the production of mAb (6D8) that protected animals from Ebola virus infection (Chen *et al.*, 2002). An Ebola immune complex (EIC) was produced by fusing Ebola glycoprotein GP1 to the C-terminus of the heavy chain of humanized 6D8 mAb that binds specifically to a linear epitope on GP1 using the geminivirus-based expression system and *Nicotiana benthamiana* (Bhoo *et al.*, 2011). The recombinant immunoglobulins were produced in leaves of *Nicotiana benthamiana*, which was purified by protein G affinity chromatography. The resultant recombinant antibody bound the the complement facto C1q, indicating immune complex formation. Therefore subcutaneous immunization of mice with purified EIC showed high level of production of anti-Ebola virus antibodies that provides protection against Ebola virus (Figure 4). This was the first published report of an Ebola virus candidate vaccine to be produced in plants.

The Ebola virus disease outbreak in West Africa has provided a great opportunity for the use of plantibodies in resolving global human health challenges

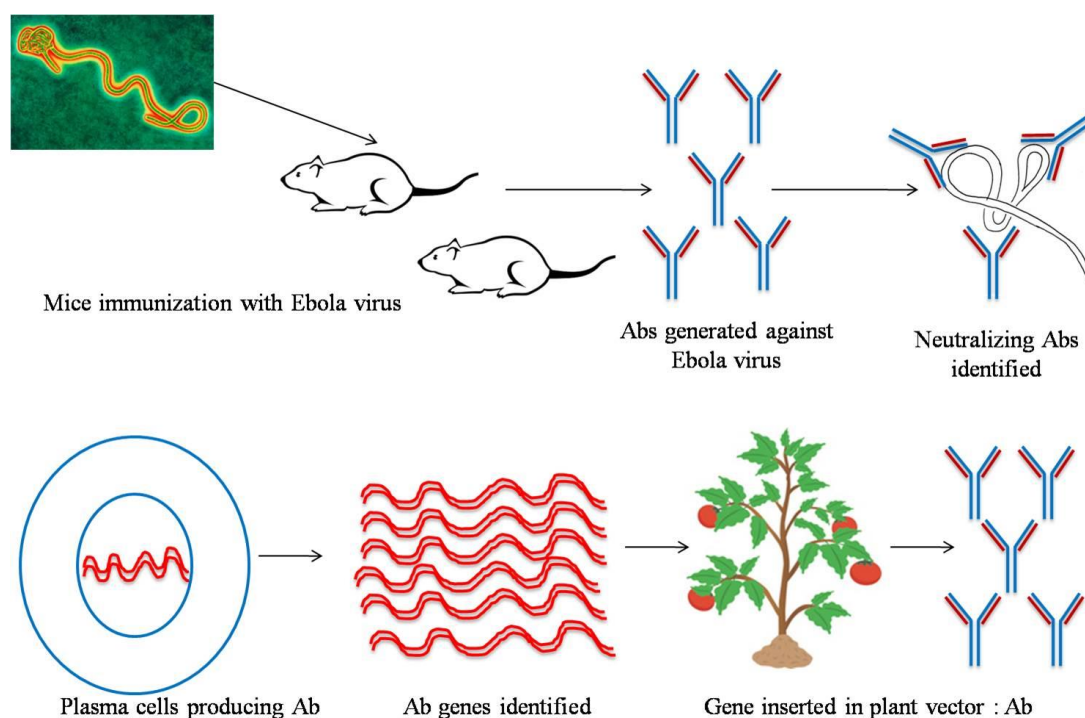


Figure 4: Use of plantibodies for Ebola virus treatment.

as two American medical aid workers who contracted the disease in Liberia were successfully treated with an experimental drug called ZMapp produced in the tobacco plant. The drug ZMapp contains combination of three humanized anti-Ebola virus mAbs and developed by Mapp biopharmaceutical incorporated, San Diego (Langreth *et al.*, 2014). Although the drug ZMapp holds great promise for the future, the major limitation is producing large quantity of anti-Ebola virus antibody to meet the demand of widespread outbreak requirement, multiple doses, direct delivery of highly pure antibody into the blood stream and moreover the drug is intended originally for expression levels sufficient for animal trial (Powell, 2014). ZMapp is yet to receive the approval by the US food and Drug Administration who have to certify that the plant extraction process has not led to the contamination of the resulting drug (Begley, 2014).

8. Advantages and challenges of plantibodies production

Plantibodies production from plants has many potential advantages for creating biopharmaceuticals related to the medicine. First, the plant systems used for plantibodies production are more economical than industrial equipments using fermentation system. Second, this system provides large amount of plant products. Third, the system can be omitted the purification step when the aim is to produce edible vaccines. Fourth, plants can be directed to the desired proteins into compartments/organelles such as chloroplasts. Fifth, the amount of proteins produced in such an amount it can be suitable for industrial levels. Last, one of the important advantage, health risks from contamination with potential human pathogens are minimized (Sala *et al.*, 2003; Daniell *et al.*, 2001).

After lots of advantages, there are some remaining challenges are associated with the plantibodies production. During

down streaming processess of plantbodies production such as extraction and purification of plantibodies is an important step, covers more than half of the total cost. Thus purification system during plantibodies production is very expensive. Currently purification system in plant systems, an affinity purification protocol requires protein A-based matrices is mainly used (Valdes *et al.*, 2003). So it is necessary to use alternative economic methods that use oleosin or polymer fusions for the purification of plantibodies (Daniell *et al.*, 2001). Till date, researches have had difficulty in achieving the high level of chloroplast gene expression. Each type of plants poses its own challenges and dosage of vaccines may be variable. Plantibodies are not suitable for infants (Doshi *et al.*, 2013).

9. Future perspectives

Plant-derived systems for production of biopharmaceutics should meet the same standards of safety and performance as other production systems (Daniell *et al.*, 2001). Plantibodies production system have many advantages over animal systems, such as well-established cultivation, quick scale-up, simple distribution by seeds, oral delivery, can be used as raw food or dry powder, desperate of cold chain requirement, mucosal and serum immune responses, cost efficiency, ease of genetic manipulation, ease of production and scale-up, safer than conventional vaccines, ideal to face bio-weapons and ideal for veterinary use as feed additive (Yoshida *et al.*, 2004; Sala *et al.*, 2003).

10. Concluding remarks

Substantial progress has been made in recent years towards the production of a wide range of antibodies in plants and now it is a feasible and economical system of producing antibodies. These transgenic plants have been shown to be the most productive system in providing therapeutics and edible vac-

cines, which are cheap and can be easily administered. Plants can be easily explored by pharmaceutical industries and Indian climate helps for the production of diversified crops. Engineering of increased pathogen resistance and alteration of phenotypes by immunomodulation provide a great platform to develop the prevention strategies. Various other strategies have been developed to exploit plants as bioreactors for the production of pharmaceutical antibodies and many plant produced antibodies are proved increased disease resistance. Recent developments focus on the detailed characterisation of recombinant products. Recent indications that tissue specific and physiological factors may have an impact on the quality and glycosylation pattern of a plant antibodies will perhaps lead to new insights and production strategies. Although some plant-derived antibody products have successfully completed early phase clinical trials, several issues including regulatory guidelines and public acceptance must still be resolved. Currently, more than 200 novel antibody-based potential products are in clinical trials worldwide, and market demand will certainly strain the capabilities of existing production systems. Moreover, adoption of plants as bioreactors on a larger scale would reduce the cost of antibody therapy and simultaneously increase the number of patients who access to these treatments.

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Renewable Energy from Agro-industrial Processing Wastes: An Overview

Sudhanshu S. Behera¹, Ramesh C. Ray^{2,*} and S. Ramachandran³

¹Department of Fisheries and Animal Resource Development, Government of Odisha, India; ²ICAR- Central Tuber Crops Research Institute (Regional Centre), Bhubaneswar 751019, India; ³Department of Biotechnology, Birla Institute of Technology and Science, Pilani, Dubai campus, Dubai International Academic City, P. O. Box No: 345055, Dubai, UAE; *Correspondence: rc.ray6@gmail.com; Tel: +91- 674- 2470528

Abstract: The continued use of fossil-derived fuels has resulted in progressive depletion of non-renewable energy resources and environmental deterioration that led to the development of an alternative renewable source of energy for sustainability. Agricultural and horticultural crops waste processing offer an option among alternatives for generating renewable energy (bio-refinery) due to its potential sustainable supply and abundance. The conversion of agro-industrial processing wastes typically contains mixed hexoses and pentoses with significant proportions of polymeric sugars (lingo-celluloses and cellulose) which are difficult to degrade and require advances in the technology. This article is highlighting the major developments in various biomass-based agro-industrial processing waste-based fuel-generating processes and suggests a possible major solution to provide energy (bio-energy) in an eco-friendly way for the reduction of greenhouse gases and pollution.

Keywords: Agro-industry; bio-ethanol; environment; processing; renewable energy

1. Introduction

The world is faced with a chronic energy crisis that has resulted in the crippling of most sectors of the economy. It is estimated that over 80% (about 450-500 EJ/year) of the world's energy demands is met by the combustion of fossil fuels (Ioelovich, 2015). The main energy sources of fossil fuels are coal, petroleum and natural gas. Coal does provide about 28% of world's consumed energy. Crude oil- petroleum, provides about 32% of world's energy while natural gas provides about 20% of the world's energy consumption (Ioelovich, 2015). However, fossil-based fuels are limited and excessive exploitation of such fossil fuel increases carbon footprints. The combustion of these fuels also caused the emission of harmful gases, including CO, CO₂, NO_x and sulfur-containing com-

pounds which are main sources of air pollution and global warming (Crutzen *et al.*, 2016). Moreover, fossil-fuel exposes the earth to changes in price of petroleum resources and political instability from the oil producing region of the world (Behera and Ray, 2014; Aliyu *et al.*, 2015).

The renewable sources of energy, such as solar, wind, and biofuel (bioethanol, bio-diesel, bio-hydrogen) have gained huge attention from governments of many countries across the world. Recently, government policies have planned to replace the petroleum based fuels with renewable biomass fuels, which are derived from agricultural residues such as sugarcane, corn, switchgrass, algae, etc (Sarkar *et al.*, 2012). These renewable resources are indigenous, non-polluting and virtually inexhaustible. Globally, more than 30% of the loss occurs from agricultural/horticultural substrates/ wast-

es (rice husk, coffee wastes, sugar cane biogases, maize harvesting wastes, and bamboo cellulose pulp, etc.) at the retail and consumer levels, of which the post-harvest and processing level wastages account for the major share (Singh *et al.*, 2012). The different residues/biomass resulting from the production of agricultural crops might contribute to the achievements of the renewable energy target as proposed for further uses (Scarlat *et al.*, 2010). The use of biomass for transport fuel, heat and electricity production will have to increase substantially to meet the proposed binding target of renewable energy in the EU energy mix of 20% by 2020 (Scarlat *et al.*, 2010). The chapter discusses the agricultural/horticultural resources and food processing wastes for potential production of bioenergy.

2. Agricultural and food processing wastes

There are various forms of agricultural and horticultural (agro-industrial) resources in the world. Among the agro-industrial wastes, horticulture processing industries form a major share throughout the world. Horticultural wastes mainly generate from fruits and vegetable processing, starchy roots and tubers, coconut, olive and palm oil mills and fruit-based fermentation industries (Panda and Ray, 2015; Panda *et al.*, 2017). The fermentation industries of solid waste include items removed from fruits and vegetables during cleaning, processing, cooking, and/or packaging. These items may include leaves, peels, pomace, skins, rinds, cores, pits, pulp, stems, seeds, twigs, and spoiled fruits and vegetables (Bouallagui *et al.*, 2005; Panda *et al.*, 2016). Globally, more than billion tons of agro-industrial and food processing solid wastes are available. The losses in industrial countries are as high as in developing countries, but in developing countries more than 40% of losses occur at post-harvest and processing levels, while in industrialized countries, more than 40% of the

losses occur at retail and consumer levels. Among them, lignocellulosic wastes derived from cereals, oil seeds, pulses and plantation crops, account for 222 million tons in industrialized countries. Fruits, vegetables and root crop processing wastes alone are available to the extent of 12 million tons (Auer *et al.*, 2017). However, over 86×10^6 t of fruits and 162×10^6 t vegetables are produced annually in India, contributing 12.6% and 14.0% of the total world production of fruits and vegetables respectively (Source FAO website- February 2014-15; Horticulture Database, India-2015). Out of the total production, nearly 76% is consumed in fresh form, while wastage, and loss account for 20-22 percent. Of which only 2-4% is processed in the fruits and vegetables processing industries. This is in sharp contrast to the extent of processing of fruits and vegetables in several other developing countries such as Brazil (70%), Malaysia (83%), Philippines (78%), and Thailand (30%) (Horticulture Database, India- 2015). Disposal of these putrescible fruits and vegetables processing wastes (organic refuse) leads to environmental and economic problems (Viswanath *et al.*, 1992; Scano *et al.*, 2014). The nutrients of food waste may be re-used in agriculture by composting or by biotransformation of food waste into animal feed and/or converted to biofuels (Table 1). These wastes contain a high amount cellulose, hemi-cellulose, and pectin, which provide a suitable substrate for fermentation process (Khalid *et al.*, 2011). These polymers can be hydrolyzed enzymatically by cellulose, β -glucosidase and patience to their corresponding soluble carbohydrates and subsequently to biofuels (Ariunbaatar *et al.*, 2014; Behera and Ray 2016).

2.1. Bio-ethanol production

Bio-ethanol has been proved to be a most promising alternate energy source with various added advantages (Manzano-Agugliaro *et al.*, 2013). The worldwide

Table 1: Current status of biofuel technologies utilizing agricultural and horticultural substrates and wastes. (Lynd *et al.*, 2015, modified)

No	Crop category	Example	Industry status
1.	Starch-rich	Maize, wheat, sorghum	About 50 billion L ethanol in the US based on maize
2.	Sugar -rich	Sugar cane, sugar beet	About 23 billion L ethanol in Brazil based on sugar cane
3.	Oil-rich	Rapeseed, soy, sunflower, palm oil	About 23 billion L produced worldwide, mostly in the EU, US and Brazil
4.	Cellulosic	Grass trees, various horticultural wastes	Liquid fuel capacity about 175 million L worldwide

bio-ethanol production is increasing constantly. The world bio-ethanol production in 2006 was 39 billion liters and has grown to 100 billion liters in 2015 (Sarkar *et al.*, 2012) and is expected to reach 180 billion liters in 2020 (Ioelovich, 2015). Brazil and USA are the two major ethanol producers accounting for 62% of the world production (Sarkar *et al.*, 2012). In Brazil ethanol is completely produced from sugar cane. In USA, the production of ethanol relies on corn starch (Ioelovich, 2015). Conventional indigenous raw materials for bio-ethanol production include sugarcane, molasses/starch and corn-based material, although the amount of bio-ethanol produced can hardly meet the current global demand (Sarkar *et al.*, 2012; Saggi and Dey, 2016). Hence, cellulosic materials such as agro-residues are attractive feedstock for bio-ethanol production. The wastes from agro-residues being rich in polysaccharides (cellulose, hemi-cellulose and lignin) have been subjected to solid state fermentation to produce bio- ethanol (Singh *et al.*, 2012).

2.1.1. Waste pre-treatment

The biomass from agro-industries are the most abundant on the earth. These biomass/substrates include corn (maize), wheat, oats, rice, potato, and cassava. On a dry basis, corn, wheat, sorghum (milo), and other grains contain around 60-70% (wt/wt) of starch, which are hydrolyzed to hexose and offered a good resource in

fermentation processes (Lin and Tanaka, 2006; Behera and Ray, 2016). However, bio-ethanol from agro-residues could be a promising technology that involves four processes of pre-treatment, enzymatic hydrolysis, fermentation and distillation (Gupta and Verma, 2015). These processes have several challenges and limitations, such as biomass transport and handling, and efficient pre-treatment process for removing the lining from the lignocellulosic agro-residues. Proper pre-treatment process may increase the concentrations of fermentable sugars after enzymatic hydrolysis, thereby improving the efficiency of the whole process. Conversion of cellulose to ethanol requires some new pre-treatment, enzymatic and fermentation technologies, to make the whole process cost effective (Gupta and Verma, 2015). However, many agricultural and horticultural wastes have low lignin content and lesser amounts of cellulose with more easily degradable polysaccharides. This results in lower enzyme pretreatment or physio-chemical costs and increased yields of fermentable sugars (Sindhu *et al.*, 2016).

2.1.2. Microflora

Most agricultural biomass/vegetable and fruit wastes (VFWs) have been used as a potential substrate for the ethanol fermentation by microbial processes. VFWs contain mainly starch, cellulose, soluble sugars and organic ac-

ids (Table 2). Microbes are well-suited natural agents for recycling of organic wastes including VFWs (Zupancic and Grilc, 2012; Panda *et al.*, 2016). The microorganisms such as *Bacillus*, *Pseudomonas*, *Rhizopus* and *Trichoderma*, well known for production of hydrolytic enzymes, are employed for biodegradation of VFWs (organic matter) to reduce the biological oxygen demand and chemical oxygen demand of the liquid wastes (Panda and Ray, 2015). More specifically, microorganisms, such as fungi (*Mucor indicus*), bacteria (*Zymomonas mobilis*), and yeasts (*Candida utilis*, *Kluyveromyces marxianus*) have been used for ethanol production from VFWs. However, yeast is most common microorganisms grown on VFWs as substrates (Stabnikova *et al.*, 2005). Among the yeast, *Candida utilis* is selected for cultivation of concentrated effluents of the food industry after its anaerobic acidogenic treatment (Stabnikova *et al.*, 2005). In contrast, *Saccharomyces cerevisiae* is the most commonly used yeast in industrial ethanol production from red beet (juice and bagasse) (Jimenez-Islas *et al.*, 2014). Moreover, biotransformation of cellulose-to-ethanol can be conducted by various anaerobic thermophilic bacteria, such as *Clostridium thermocellum*, and some filamentous fungi, including *Monilia* sp., *Neurospora* sp., *Zygosaccharomyces rouxii*, *Aspergillus* sp., *Paecilomyces* sp., and *Trichoderma viride* (Lin and Tanaka, 2006). Others efficient microbes and genetically modified microbes may also enhance the enzymatic hydrolysis. The approach of using depolymerizing enzymes producing co-culture or construction of engineered microorganisms are attractive for low capital cost and enhanced yield of fermentable sugars (Arora *et al.*, 2015).

2.2. Fermentation process

The varied raw materials used in the fermentation are classified into three categories: sugars, starches, and cellulose materials (Mohapatra *et al.* 2017). The main sources of sugars (sugar cane, sugar

beet, molasses and fruits) converted into ethanol directly. The starches (corn, cassava, potatoes, and root crops) are initially hydrolyzed to fermentable sugars by the action of enzymes (Ray and Naskar, 2007; Ray *et al.*, 2008). Celluloses (agricultural residues, wood, waste sulfite liquor from pulp and paper mills) are converted into sugars by the action of mineral acids. The simple sugar so formed, can readily fermented to ethanol by the action of microbial enzymes (Lin and Tanaka, 2006; Monlauet *et al.*, 2013).

2.2.1. Anaerobic digestion and methanogenesis

Anaerobic digestion is a biochemical degradation process that is widely used for the treatment and energy recovery from many kinds of biomass, especially agricultural products and agro-industrial wastes (Scano *et al.*, 2014). Hydrolysis and acidification are the advantages in two-phase in anaerobic digestion processes. FVWs are characterized by a high percentage of moisture (>80%), high organic content (volatile solids>95% of total solids), are readily biodegraded and are therefore suited to energy recovery through anaerobic digestion (Jiang *et al.*, 2012). Scano *et al.* (2014) studied the biogas production through an anaerobic digestion pilot plant by using VFWs as single substrate. The experimental study was carried out using most suitable operating parameters and optimum organic loading rate was reported at 2.5-3.0 kg volatile solids/m³d with maximum biogas production of 0.78 Nm³/kg volatile solids (CH₄ content of 55%).

2.3. Factors affecting biogas production

Various aspects/factors influence the biogas production from the feed stocks. The C: N ratio, pH, temperature, total solid contents, organic loading rates, hydraulic retention time, design of digester, inoculum quality and volatile fatty acid content also regulate and impact biogas production.

Table 2: Bio-energy production from lignocellulosic material by various microorganisms

Microorganisms	Waste substrate	Feature of the employed microorganism	Bio-energy production	References
Bacteria				
<i>Pichia stipites</i> BCC15191	Sugar cane bagasse	Ferment both glucose and xylose	Bio-ethanol; 0.92g/g	Buaban et al. (2010)
<i>Pichia stipitis</i> DSM 3651	Wheat straw hydrolysates	-	Bio-ethanol; 0.41 g/g	Bellido et al. (2013)
<i>Clostridium acetobutylicum</i>	Rice straw	Ferment mono-, poly-saccharides	ABE; 10.5 g/L	Amiri et al. (2014)
<i>C. beijerinckii</i>	Wheat straw hydrolysates	Ferment hexose and pentose	ABE; 11.44 g/L	Bellido et al. (2014)
<i>Kluyveromyces marxianus</i> ATCC 36907	Sunflower biomass	Cellulase, & β -glucosidase activity	Bio-ethanol; 27.88 g/L	Camargo et al. (2014)
Yeasts				
<i>Saccharomyces cerevisiae</i> IBB10B05	Spent sulfite liquor	Ferment hexose and pentose	Bio-ethanol; 0.31-0.44g/g	Novy et al. (2013)
<i>Saccharomyces cerevisiae</i>	Banana & orange peels	Improved cellulase activity	Bio-ethanol; 28.6-40.7 g/L	Singh et al. (2014)
Co-culture strains				
<i>Saccharomyces</i> sp. W0 + <i>Pichia pastoris</i> X-33/pPICZaA- <i>INU1</i>	Tuber meal of Jerusalem artichoke	Recombinant inulinase	Bio-ethanol; 0.319g/g	Zhang et al. (2010)
<i>Candida shehatae</i> + <i>Saccharomyces cerevisiae</i>	Sugarcane bagasse	Improved cellulase activity	Bio-ethanol; 3.2g/L	Chandel et al. (2013)
<i>Bacillus subtilis</i> and <i>Pseudomonas aeruginosa</i>	Orange peel	Improved cellulase activity	Bio-ethanol; 82.7-92.2 g/L	Gomaa, (2013)
<i>Trichoderma reesei</i> + <i>Saccharomyces cerevisiae</i>	Oil palm EFB	Improved cellulase activity	Bio-ethanol; 4.6 mg/mL	Karim et al. (2014)
Filamentous fungi				
<i>Fusarium oxysporum</i>	Glucose, birchwoodxylan, corn cob or wheat bran	Over-expression of xylanase	Bio-ethanol; 2.68-2.85 g/L	Anasontziset al. (2011)
<i>Trichoderma</i> sp. + <i>Penicillium</i> sp. + <i>Aspergillus</i> sp.	Sugarcane bagasse and corn-cob	Over-produced cellulases*	Bio-ethanol; 58 g/L	El-Bondkly and El-Gendy, (2012)
Recombinant (micro) organisms				
<i>Saccharomyces cerevisiae</i> 424A	Corn stover	Ferment glucose and xylose	Bio-ethanol; 40 g/L	Lau and Dale, (2009)

Table 2: Continued..
(LNH-ST)

<i>Escherichia coli</i> FBR5	Wheat straw hydrolysate	Ferment hexose and pentose	Bio-ethanol; 8.9-17.3 g/L	Saha and Cotta, (2011)
<i>Escherichia coli</i> FBR5	Lignocellulosic biomass	Ferment hexose and pentose	Bio-ethanol; 50 g/L	Cotta, (2012)
Immobilized (micro) organisms				
<i>Zymomonas mobilis</i> MTCC 92	Sugarcane mo- lasses	Entrapped in luffa sponge disc & Ca- alginate beads	Bio-ethanol; 58.7-59.1 g/L	Behera et al. (2012)
<i>Saccharomyces</i> <i>cerevisiae</i> MTCC 3090	Molasses	Immobilized sodium-alginate	Bio-ethanol; 7.6 g/L	Agnihotry et al. (2015)

ABE: Acetone-butanol-ethanol; EFB: Empty fruit bunches; Luffa: *Luffa cylindrica* L.; Over produced cellulase*: exo- β -1,4-glucanase, endo- β -1,4-glucanase and β -1,4-glucosidase

2.3.1. Carbon to nitrogen (C: N) ratio

A C/N ration between 22 and 25 seemed to be better for anaerobic co-digestion of FVWs with its co-substrates. The most significant factor for enhanced FVWs digestion performance is the improved organic nitrogen content provided by the additional wastes (Bouallagui et al., 2009).

2.3.2. PH

The optimum pH and pH range differs with substrate and bio methanation technique. The optimum pH values for the acidogenesis and methanogenesis stages are different. During acidogenesis stage, lactic, propanoic, and acetic acids are formed and thus, the pH falls. The low pH (about 6.4) can be toxic for methane-forming bacteria (optimum between 6.6-7.0). Most of digested carbohydrate substrates are acidic and developing pH of 6.2 or less and thus becoming toxic (Chanakya and Malayil, 2012). A suitable amount of lime is added to neutralize the acid accumulated in the carbohydrate residues of processing digesters.

2.3.3. Temperature

The temperature is an important parameter for bio methanation. Tempera-

ture has significant effect on the microbial community, process kinetics and stability and methane yield (Patil and Deshmukh, 2015). The high temperature cooking (140-180°C) is very effective for fermentation of ethanol from starchy material in industrial-scale. The high temperature enhances the efficiency of starch saccharification and achieves high levels of ethanol under complete sterilization of harmful microorganisms (Krishania et al., 2013). However, to resolve the difficulties of high production costs and requirement of additional amounts of enzymes (amylase), non-cooking and low-temperature cooking fermentation system have been recently developed (Ghimire et al., 2015).

2.3.4. Total solid content

Total solid content influences biogas production from fruits and vegetable wastes. Abbassi-Guendouz et al. (2012) investigated the role of total solid content in anaerobic digestion in batch reactors.

2.3.5. Organic loading rate /Volatile solids

The organic loading rate determines the input of organic matter per unit volume of digester capacity per day

measured of the biological conversion capacity of the anaerobic digestion system (Patil and Deshmukh, 2015). There is an optimum feed rate for a size of digester is essential for optimum yield of biogas (Patil and Deshmukh, 2015). Shen *et al.* (2013) performed the anaerobic co-digestion of FVWs and food waste in single-phase and two-phase digesters at various organic loading rate ($3.5\text{--}5.0\text{kg. Volatile solids. m}^{-3}. \text{d}^{-1}$) to investigate biomethane production ($0.328\text{--}0.544\text{m}^3. \text{kg}^{-1}. \text{Volatile solids}$).

2.3.6. Hydraulic retention time

The amount of time the feedstock stays in the digester is known as hydraulic retention time the retention time must be sufficient to carry out the necessary degree of biodegradation (Patil and Deshmukh, 2015). Bio methanation of banana peel and pineapple wastes studied at various hydraulic retention times showed a higher rate of gas production at lower retention time (Velmurugan and Ramanujam, 2011). The lowest possible hydraulic retention time for banana peel was 25 days, resulting maximum rate of gas production of 0.76 vol/vol/day with 36% substrate utilization, while pineapple-processing waste digesters was operated at 10 days' hydraulic retention time, with a maximum rate of gas production of 0.93 vol/vol/day and 58% substrate utilization (Hosseini and Abdul Wahid, 2014). To maximize the yield of biogas and to improve its quality (high CH_4 content and low H_2S content) different strategies can be followed: 1) daily organic loading rate must be kept constant 2) use of well balanced mix of feeding substrate/wastes 3) two stages process to separate the hydrolysis and acidogenesis phases from methanogenesis phase (Scano *et al.*, 2014).

2.3.7. Digester design

Various kinds of digesters are used for anaerobic process such as one-stage or two-stage digester, wet or dry digesters, batch or continuous process di-

gesters, high rate digesters or digesters with combination of different approaches for bioenergy (Ganesh *et al.*, 2014). However, most commonly used techniques of bio-hydrogen production, including direct bio-photolysis, indirect bio-photolysis, photo-fermentation and dark-fermentation and conventional or modern techniques (Mudroom *et al.*, 2011).

3. Anaerobic digestion process from fruit and vegetable wastes

Normally, biogas is composed of 45-70% methane, 30-45% carbon dioxide, 0.5-1.0% hydrogen sulfide, 1-5% water vapor, and a small amount of other gases (hydrogen, ammonia, nitrogen, etc.). However, the composition varies with the sources of biodegradable biomass. Biomethane, obtained during anaerobic digestion by the microbial community of biodegradable agricultural and horticultural substrates/wastes (Singh *et al.*, 2012).

3.1. Ensiling and methane generation

Ensiling techniques is the process of bio methanation using the storing of forage crops and various other agricultural commodities such as mango peel, orange, lemon and lime peels, pineapple and tomato processing wastes for a prolong period (Kreuger *et al.*, 2011; Panda *et al.*, 2017). Effects of ensiling process, storage of biological/agricultural silage additives are attributed to increases in organic acids and alcohols contents and showed positive effects on methane yield (Herrmann *et al.*, 2011). Several processes have been developed for high rate bio-methanation. The processes include: 1) up-flow anaerobic sludge blanket, 2) expanded granular sludge bed, 3) fixed film, 4) fluidized bed and 5) plug flow. Fang *et al.* (2011) operated the up-flow anaerobic sludge blanket reactor using the potato juice for biogas production. The methane potential was determined at the highest organic loading rates of $5.1 \text{ g COD. (L-reactor. d)}$

with the methane yield of 240 mL-CH₄/g volatile solids-added.

3.2. Acetone-butanol-ethanol production

There is also renewed interest in reviving the acetone-butanol-ethanol process through application of the recombinant strains (Lütke-Eversloh and Bahl, 2011) and process development and using cheaper agricultural wastes/substrates (Green, 2011). The bio-conversion of lignocellulosic substrate/wastes to monomeric sugars and its consequent fermentation has been suggested for economic production of acetone-butanol-ethanol (Amiriet al., 2014). A variety of bacterial strains, such as *Clostridium aurantibutyricum*, *C. beijerinckii* and *C. butyricum* participate in acetone-butanol-ethanol production and utilize a variety of substrates including pentose, hexose, starch, and xylan but not cellulose (Bellido et al., 2014). Further, development can be directed by manipulating and controlling the fermentation conditions by reducing the toxic effect of products (repression) on cell physiology and promoting one dominant solvent product during production of acetone-butanol-ethanol.

3.3. Microbial hydrogen production

Hydrogen is produced by several processes, such as electrolysis of water, thermocatalytic reformation of hydrogen-rich organic compounds, and biological processes. Currently, biological production of hydrogen (bio-hydrogen) from horticultural residues, using microorganisms, is an exciting new area of technology development (Levin et al., 2004). Asian countries possess significant potential for producing bio-hydrogen from crop residues. Bio-hydrogen production by culture of bacteria is highly attractive for larger-scale applications (Kumar et al., 2015). Microbes, including strict anaerobes (clostridia, ruminococci and archaea) and facultative anaerobes, including *Escherichia coli* and *Enterobacter aerogenes* and aerobes, including *Alcali-*

genes eutrophus and *Bacillus licheniformis* when held under anoxic conditions, can produce hydrogen from organic substrates/wastes (Sivagurunathan et al., 2016).

3.4. Biodiesel

The technology implemented for production of liquid biofuels is based on transformation of food-grade biomass (carbohydrates) into bioethanol and vegetable oils into biodiesel fuel. The main sources of juices of sugar cane, sugar beet, and sweet sorghum, as well as starches of corn, wheat, potato, and some other agricultural plants (Ioelovich, 2015). Oil-seed crops are the largest sources of exploitable biomass to produce liquid fuel, bio-diesel (i.e., fatty esters). Bio-diesel offers enhanced safety characteristics as compared to diesel fuel, having no emission of explosive air/fuel vapors (Bhuiya et al., 2014; Kumar and Sharma, 2015). Considerable research has been progressed on the use of vegetable oils as diesel fuel. Vegetable oils such as soybean oil, sunflower oil, coconut oil, rapeseed oil, Tung oil, and palm oil are the best choice (Carlsson, 2009). The most common way to produce bio-diesel is by transesterification, which refers to a catalyzed chemical reaction of vegetable oil and an alcohol to yield fatty acid alkyl esters (i.e., biodiesel) and glycerol (Shahid and Jamal, 2011). Indigenous to central-south America, *Jatropha* was introduced to Africa a few centuries ago. It is currently widely distributed throughout these areas where rural inhabitants generally make extensive use of it. Oil from the seeds of *jatropha* is used as a bio-diesel substitute (Osseweijer et al., 2015).

4. Challenges and further prospective

The production of bioenergy and food production is interrelated and is affected by global change of atmospheric (rising CO₂ and tropospheric ozone), climate (temperature and soil moisture), and land degradation (saliniza-

tion, desertification, fertility loss) (Osseweijer *et al.*, 2015). Recently, global energy crisis needs optimum yield of bioenergy from advanced fermentation technology converting residues/substrates from agro-industries into ethanol, enzyme technology for hydrolysis of lignocellulosic materials, immobilization of microorganisms in pilot-scale for production of bio-energy. Furthermore, C4-type crops possess the features of high photosynthetic yield, high rate of CO₂ fixation, produce more biomass, and resistance to aridity when compared with C3 crops. Therefore, C4 type of crops are to be more investigated and need to be focused for further bio-energy production (Koçar and Civaş, 2013).

5. Concluding remarks

To date, bio-fuel has been evolved from first to fourth generation and they are mainly differed in feedstock and production technologies. The agricultural and horticultural residues based energy crops are critical and needs to be investigated as raw materials for bio-fuels for today and for the future demand. To attain the highest sustainability in bio-fuel production, continuous research and development on all sustainability-aspects is essential.

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Mitigation of Climatic Change by Organic Agriculture

Mohan Mani^{1,*}, Manohar Murugan², Ganesh Punamalai³ and
Vijayalakshmi Ganesan Singaravelu⁴

¹Mahendra Engineering College(Autonomous), Namakkal, Tamil Nadu, India;

²Vivekananda College of Arts and Science (Autonomous), Elayampalayam, Namakkal Dt., Tamil Nadu, India; ³Department of Microbiology, Faculty of Science, Annamalai University, Chithamparam, Tamil Nadu, India; ⁴Former Professor of Environmental Biotechnology, Manonmaniam Sundaranar University, Tirunelveli, Tamil Nadu, India;

*Correspondence: mohanrtt@gmail.com; Tel: +91-9486069246

Abstract: Agricultural inputs and farming systems are playing vital role in the consumption of fossil fuels and climate change. Organic and non-organic production in terms of energy use is essential for understanding the energy inefficiencies of different agro systems and their potential for minimizing energy consumption and mitigating environmental impacts particularly climate change. Organic agriculture can provide a more energy efficient approach which leads to sustainable agricultural productions. Organic agro productions is contributing less greenhouse gas emissions and have a superior potential to sequester carbon available in biomass than conventional agro production. The energy efficiency of organic agriculture in terms of bioenergy production and thereby renewable fuel source is to reduce dependency of fossil fuel energy and mitigate environmental pollution caused by emissions. The industrialized production of agro products is responsible for a heavy impact on the environment and playing a major role in increasing global Green House Gas (GHG) emissions in respect to the usage of synthetic fertilizers and pesticides. More than 480 million tons of GHG is released to the atmosphere each year by the synthetic fertilizers and pesticides factories. Climate change is a serious environmental issue and has broad impacts on sustainable development and the future of our economy, health, and agricultural production sector. Integrated farm management system is reducing and sequestering GHG emissions. Organic farming is essential to ensure the future of our environment and food production in a sustainable manner. This article highlights the importance of organic agriculture and its role in mitigating climatic change.

Keywords: Carbon sequestration; climatic change; greenhouse gases; organic farming; sustainable production

1. Introduction

Agriculture in India is the promising sector for millions of livelihood around two thirds of the work force in the country. Most of the population is dependent on agriculture and allied sectors which contribute nearly 24 per cent of gross domestic product (GDP) of India (Mahadevan, 2003). Crop production has changed significantly in the past decades

and agricultural practices highly dependent on synthetic pesticides and fertilizers. Equipment and machinery for cultivation is mainly dependent on consumption of fossil fuels. The modern agricultural practices and industrialization of agro system has created adverse effects on the environment and thereby emitting more Green House Gases (GHG). The main factor in anthropogenic climate change is the increase in the concentration of carbon in

the atmosphere over time. This increased concentration has been caused by the emission of GHGs as a result of economic activities, including energy, industry, transport, and land use, many of which rely upon fossil fuels (Banuri and Opschoor, 2007). Climatic change is influencing the food caching behavior of a wide range of animals to store food for future use. (Sutton *et al.*, 2016). Migrating animals are vulnerable to climate change and it affects the migratory bird movement. (Seebacher and Post, 2015). Mollusks, and coral reefs are observed negative impacts of climatic change (Tschakert 2015). Prolonged exposures to elevated CO₂ have affected structural proteins like actin (Ertl *et al.*, 2016). The core objective of the this chapter is to elucidate the importance of organic farming as the alternative to modern farming system and leads to the sustainable agricultural system to overcome the recent changes in the environment and energy crisis faced by the present generation.

2. Greenhouse gas emissions from agriculture

Agricultural activities are responsible for the emission and sinks for greenhouse gases. The industrial production of nitrogen based fertilizers, the combustion of fossil fuels are the primary sources of greenhouse gases released from agriculture. Enteric fermentation or

the fermentation that takes place in the digestive tract of ruminants observed in livestock resulted the emission of 40% of CO₂eq (Figure 1). Photosynthesis is the natural process in which the CO₂ is converted to organic carbon and it is converted to CO₂ by respiration and decomposition. Carbon restoration to the soil is happened by many agricultural practices include, conservation tillage, recycling agricultural residues, cover cropping and crop rotations. Biological carbon sequestration is a process by which the greenhouse gases generated during agricultural activities have been removed from the atmosphere. Agriculture recorded from 10 to 12 percent of total global human caused emissions of greenhouse gases during 2005, according to the report by Intergovernmental Panel on Climate Change (IPCC, 2007). India is recorded the second largest emitter of CO₂ from total agricultural practices and by the application of the synthetic fertilizers followed by China (Figure 2 and 3).

3. Organic agriculture and climatic change

Organic farming is the time immemorial practice in India and it was a part of the traditional farming systems. The ancient history of knowledge like the Vrikshayurveda, Agnipurana, Brihat Samhita and Arthasasthra is exemplifying the traditional Indian agriculture. Traditional

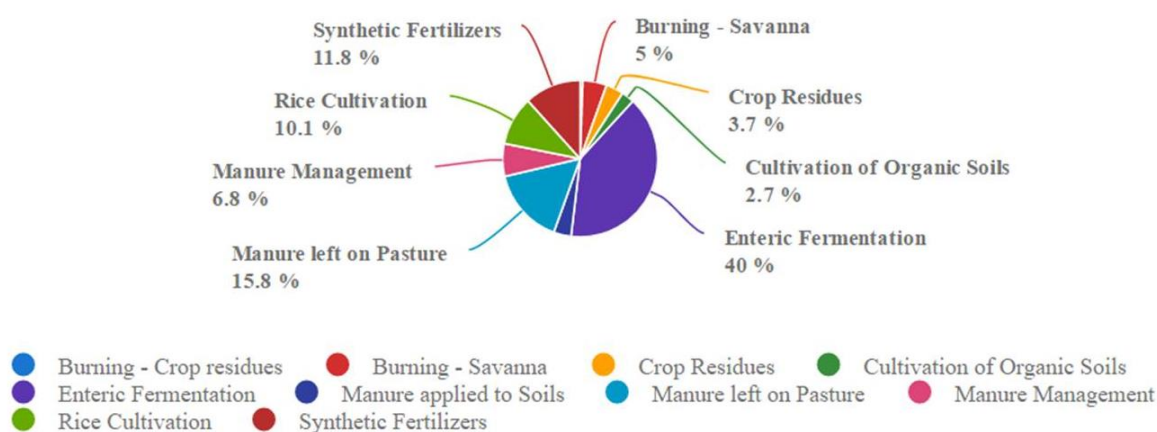


Figure 1: Average CO₂ emission by sector observed during the year 2000 and 2014 (Source: FAOSTAT 2014).

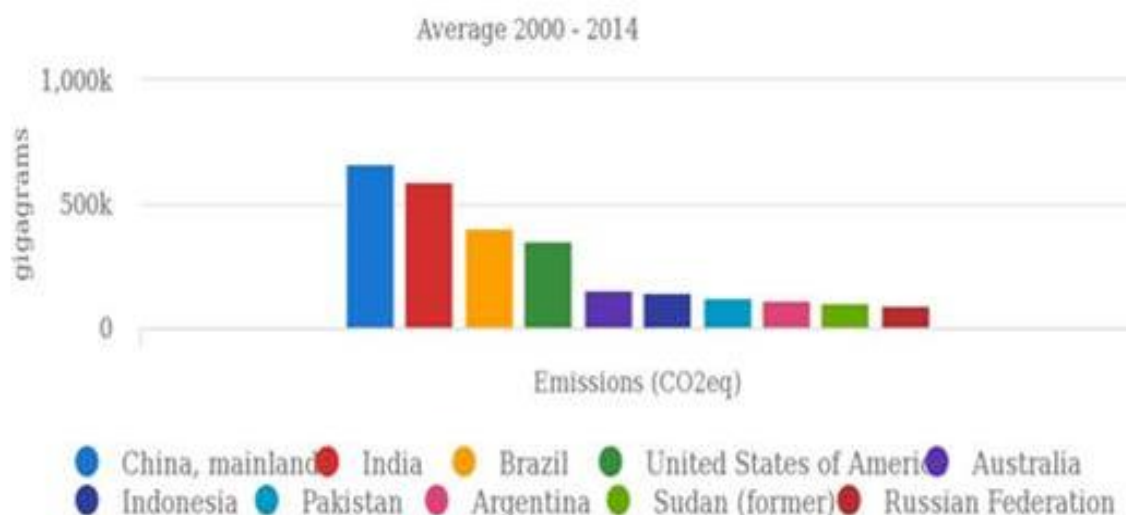


Figure 2: Top 10 CO₂ emitters during agricultural production (Source: FAOSTAT 2014).

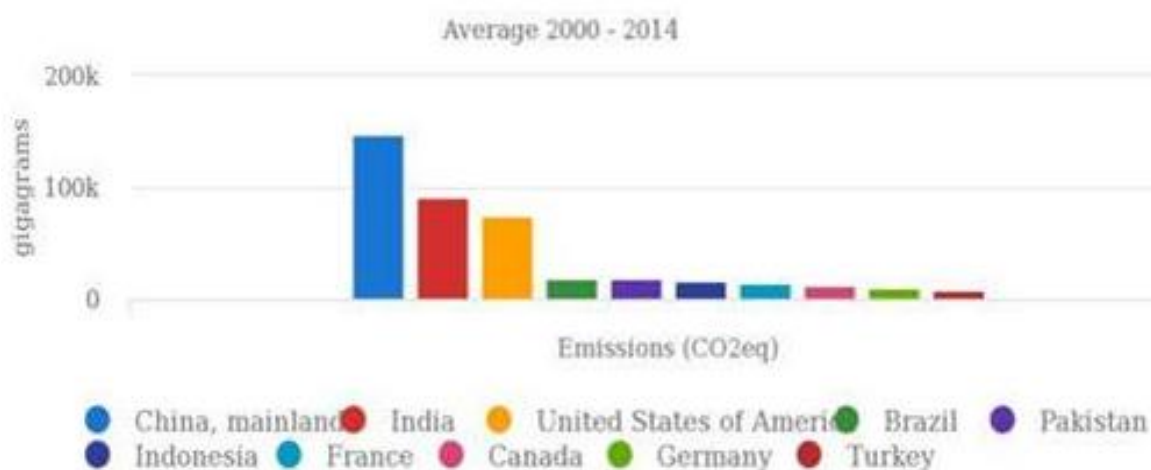


Figure 3: Top 10 CO₂ emitters by the application of synthetic fertilizers (Source: FAOSTAT 2014).

farmers who are doing organic agriculture were efficiently utilized the land resource and appropriately utilizing the SOIL (Soul Of Infinite Life).

Organic agriculture is a holistic production management system that avoids use of synthetic fertilizers, pesticides and genetically modified organisms, minimizes the pollution of air, soil and water and optimizes the health and productivity of interdependent communities of plants, animals and people (Codex Alimentarius Commission, 2001).

Organic agriculture is playing a significant role in climate change and food security to the world's population. Climate change mitigation and food security are vital management mediated by organic

farming practices. The demand for food and renewable energy sources are increased with the increase of population. The impacts of climate change by the usage of fossil fuel supported chemical fertilizers, herbicides and pesticides will create deleterious issue on agricultural production.

Organic agriculture is proved to be a remedy for climate change and the global food insecurity and potentially solve the issues related to vulnerability, unsustainability and social inequity of agricultural production. Climate change has its most significant impacts on agriculture because of its broad geographic dispersion and highly dependent on climate and environmental factors. Developing countries

are the least responsible for climate change, yet the most at risk from its effects (UNESCO, 2010). There is a need of a policies and practices to protect the ever changing environmental conditions and ecosystems that ensure the sustainable development. Organic agriculture is effectively retaining soil organic matter, soil carbon and there by balancing of ecosystem. Organic agriculture is the traditional practice that mitigates climate change, generate evergreen farming systems, eradicate poverty and augment the level of food security. Organic agriculture discharges very minimum levels of greenhouse gases (GHG) and efficiently sequesters carbon in the soil. Organic agriculture makes faming system and people dependent on this sector more resilient to climate change due to its water use efficiency, tolerance to extreme weather conditions and lower or no risk of total crop failure. Organic agriculture is extensively practiced agro-ecological farming system that accomplishes the main objectives of enabling people to thrive while improving our eco-systems and their natural cycling process.

4. Soils for food security and climate - 4 per 1000 initiative

The 21st Conference of the Parties (COP21) of the United Nations Frame-

work Convention on Climate Change (UNFCCC) held in Paris in December 2015 focused to lower the greenhouse gas (GHG) by launching a voluntary action plan named 4 per 1000 initiative - Soils for food security and climate (4o/oo Initiative). It initiated to increase soil carbon stock annually by 0.04 % through carbon sequestration. The annual growth rate 4 parts per thousand of the soil carbon stock would make it possible to stop the present increase in atmospheric CO₂. Food security is confirmed by providing proven technologies to the farmers in developing countries to repair and sustain the soils.

To feed 9.5 billion in 2050, it is essential to keep our soils alive. Figure 4 shows various steps to ensure soil organic matter by providing various ecosystem services like capable of resisting the soil from erosion, retaining water, increasing soil fertility and proliferating soil biodiversity as per the initiation suggested by 4 per 1000. It can meet three fold challenges including food security, adaptation to food system and people to climate change and mitigation of anthropogenic emission. Soils under organic management will often increase the level of soil organic carbon more than conventional management (FAO 2011 and 2011a).

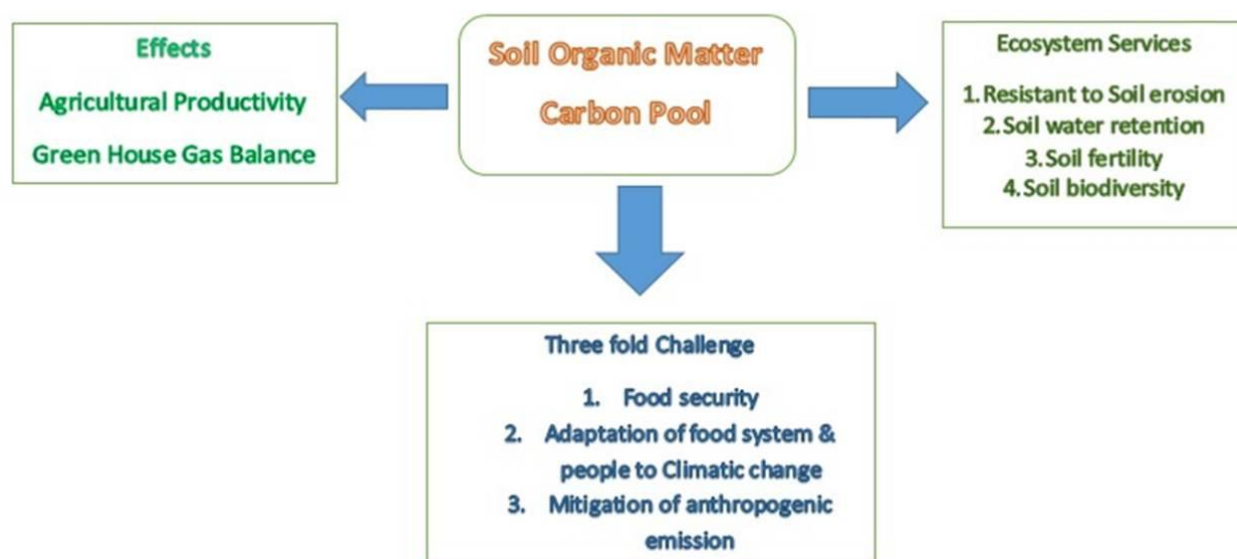


Figure 4: Ecosystem services by soil organic matter and its effects and challenges.

5. Climatic change – Indian scenario

India is holding 2.4% world surface area, 17.5% of world's population and is the fastest growing major economy in the world. It is the fourth largest greenhouse gas emitter, accounting for 5.8 % of global emissions. India's emissions increased by 67.1 % between 1990 and 2012, and are projected to increase up to 85 % by 2030 (MoEF, 2014). Increase in temperature beyond critical limits by increased industrialization resulted to reductions in rice, sorghum and maize yield (Saravanakumar, 2015). The climatic change includes changes in the intensity and distribution of rainfall and elevation of the level of oceans and a growing increase in the frequency and intensity of extreme climatic phenomena (Escobar, 2009).

India has abundance source of solar, wind, hydro power and more potential for bioenergy production. Sustainable development can be achieved by the integration of bioenergy sector with agricultural practices. Various regulatory frameworks are formed by India for the developmental strategies for encouraging bioenergy and sustainable agriculture. Biomass potential in integration with agriculture for power generation and climate change mitigation in Indian scenario is essential for the current situation (Kothari et.al, 2015).

The contributions of our country will take in to account the imperatives for addressing the challenges of poverty eradication, food security and nutrition, universal access to education and health, gender equality and women empowerment, water and sanitation, energy, employment, sustainable cities and human settlement and last but not the least, the means of implementation for the following enhanced action for achieving among others sustainable development goals.

- Jawaharlal Nehru National Solar Mission
- National Mission for Enhanced Energy Efficiency

- National Mission on Sustainable Habitat
- National Water Mission
- National Mission on Sustainable Agriculture
- National Mission for Sustaining the Himalayan Ecosystem
- National Mission for Green India
- National Mission on Strategic Knowledge for Climate Change
- State Action Plan on Climate Change
- Auto Fuel Vision and Policy 2025
- Indian Network for Climate Change Assessment

An Expert Committee was constituted by the Government of India and have recommended a roadmap for improving auto fuel quality in India till 2025 and provided vehicular emission norms for various categories of vehicles. As a result, a roadmap for rolling out Bharat Stage-IV (BS-IV), equivalent of Euro-IV, by 2017 and BS-V (Euro-V) auto fuels by 2020 in the entire country was recommended for implementation (MoEF, 2014).

6. Sustainable development and climatic change

Sustainable development, defined as “*development that meets the needs of the present without compromising the ability of the future generations to meet their own needs*” (WCED, 1987). It involves a harmonious incorporation of a viable economy, responsible governance, people's empowerment, social consistency and ecological balance. Sustainable development means economic development with strong correlation with the improvement of environmental quality. Economic development and maintaining environmental quality are essential for sustainable development.

Sustainable development is an effective tool for mitigation and adaptation from climate change, a major constraint faced by recent years. Eriksen et al. (2011) have charted out four principles

that can guide adaptation responses in a manner that supports sustainability. Sustainable adaptation should (1) recognize the context of vulnerability, including multiple stressors, (2) acknowledge that different values and interests affect adaptation outcomes, (3) integrate local knowledge into adaptation responses and (4) consider potential feedbacks between local and global processes.

7. Concluding remarks

This chapter highlights that the organic farming system is an efficient tool for tackling the climatic change mitigation and energy efficient pathway for fulfil the goal of food and energy for all. Both food security and energy efficiency are consistently managed by organic agricultural practices. It is essential to mitigate the climate change and shift from modern agricultural practice to organic agriculture is an alternative that can conserve energy with environment protection without compromising the requirements of the human beings. It is essential to consider that organic agriculture will be the solution for the problem faced by the present agricultural practices. The global solution is necessary at this juncture to overcome the impact caused by climatic change. Adopting organic agricultural practices globally for the sustainability will boost the chances of achieving 2°C target and minimize the temperature below 1.5°C.

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Application of Anti-vibrio and Anti-quorum Sensing Technology for Sustainable Development in Shrimp Aquaculture

Ramesh Kandasamy^{1,*}, Amutha Raju² and Manohar Murugan¹

¹Department of Microbiology, Vivekanandha College of Arts and Sciences for Women (Autonomous), Elayampalayam - 637 205, Tiruchengode, Namakkal Dt., Tamil Nadu, India;

²Department of Biotechnology, Periyar University PG Extension Centre, Dharmapuri, Tamil Nadu, India; *Correspondence: ksrames@gmail.com; Tel: +91 9943112125

Abstract: Farming of various marine shrimp species has been developed and commercialized in the last three decades. In the beginning, marine shrimp were cultivated in South-east Asia by farmers who raised them as incidental crops in tidal fish ponds. Over the years, enormous progress in developing shrimp culture techniques has been made. Shrimp culture evolved from an extensive farm using tidal zones to a super-intensive one in the 2000's using ponds more inland. Outbreak of disease such as Vibriosis is considered as one of the important constraint and challenge in aquaculture industry of the world. Conventional approaches such as the use of antibiotics, disinfectants and other antimicrobial drugs have shown limited success in the disease prevention. In this context, there are sound reasons for studying the beneficial eco-friendly actions of probiotics and phyto-medicine in boosting the shrimp aquaculture production without or minimal adverse impacts on environment. This chapter highlights the anti-vibrio and anti-QS nature of probionts and plants against the *Vibrio* pathogen of luminous Vibriosis, a major bacterial disease in shrimp aquaculture.

Keywords: Anti-vibrio; *Penaeus monodon*; probiotics; quorum sensing; *Vibrio harveyi*

1. Introduction

Aquaculture is one which comprises all forms of culturing aquatic animals and plants in fresh, brackish and marine environments. According to FAO statistics, aquaculture is one of the fastest growing food producing industries. It has increased at an average rate of 8.9% per year since 1970. In developing countries, in addition to agriculture the exploitation of aquatic resources can provide additional animal protein. Aquaculture can be an excellent complement to meet the food requirement of growing population. Further, it is estimated that half of the world's seafood demand will be met by aquaculture in 2020. Shrimp aquaculture is widespread throughout the tropical world. Currently,

there are about 68 countries having shrimp farm operations. Among them, 22 countries reported producing *Litopenaeus vannamei* (white shrimp), while 23 countries are producing *P. monodon* (black tiger shrimp). In 2002, the global shrimp farming industry produced an estimate of 1.6 million metric tons of shrimp and production is projected to increase at a rate of 12-15% per year over the next several years (Rosenberry, 2003). World shrimp aquaculture production has grown tremendously from a production of 200,000 tons in 1985 to approximately 3.8 million metric tons in 2012 (GOAL 2011 shrimp aquaculture survey). In 2002, the global shrimp farming industry produced an estimate of 1.6 million metric tons of shrimp and production is projected to increase at a rate of

12-15% per year over the next several years (Rosenberry, 2003). The black tiger shrimp, *Penaeus monodon* and the Pacific white shrimp, *Litopenaeus vannamei* are the most widely cultured species everywhere. Currently, there are about 68 countries having shrimp farm operations. Among them, 22 countries reported producing *Litopenaeus vannamei* (white shrimp), while 23 countries are producing *P. monodon* (black tiger shrimp). But as with many other industries, rapid growth of this sector has brought with it the problem of environmental pollution. Though the shrimp hatchery technology has advanced over the decades, the hatchery production is frequently affected by viral and bacterial disease inflicting huge loss.

2. Life cycle of penaeid shrimp

Penaeid shrimp (*Penaeus monodon*) is otherwise called Giant black tiger shrimp because of its huge size and tiger-striped band appearance in the tail. Penaeid shrimp belong to the largest phylum in the animal kingdom, the Arthropoda. This group of animal is characterized by the presence of paired appendages and a protective cuticle or exoskeleton that covers the whole animal. Presence of cephalothorax with stiff rostrum and segmented abdomen on the external of the animal is unique characters which distinguished from other species. Organs like heart, gills and digestive tract are to be found in Cephalothorax. In the head region, antennules and antennae perform sensory functions. The mandibles and the two pairs of maxillae form the jaw-like structures that are involved in food uptake. In the thorax region, the maxillipeds are the first three pairs of appendages, modified for food handling and the remaining five pairs are the walking legs (pereiopods). Five pairs of swimming legs (pleopods) are found on the abdomen.

Life cycle of a typical penaeid species includes several stages in different habitats. Mangrove estuaries and brackish water provide suitable environment for the

growth of Juveniles. Adults prefer high salinity for reproduction; hence they migrate into deep shore where the mating takes place. About 50,000 - 1,000,000 eggs are laid by female per spawning (Rosenberry, 1997). The eggs hatched out and release the first stage of larvae called nauplius. After a few days they develop into the protozoeae and metamorphose into mysids. The mysids develop into postlarvae (PLs), a stage of megalopas and share most of the adult characters. As they become larvae, migrate into offshore plankton-rich surface water. PL reaches 330 mm or above in length and 25-30 g in weight within 3-4 months after stocking in culture ponds with wide range of salinity (Lee and Wickins, 1992; Rosenberry, 1997). This rapid growth of *P. monodon* made to initiate many culturing industries near the coastal area. Ultimately it leads to generate crowding and environmental degradation that make the rearing animal more susceptible to various diseases (Johnson, 1989).

3. Factors influencing shrimp health

Many factors influence shrimp health status such as the age of shrimp, management conditions, biotic and abiotic stress and pathogens (Figure 1). Infectious disease is one of the major limiting factors in shrimp farming. Shrimp can be threatened by protozoan, fungal, bacterial and viral pathogens but viral and bacterial diseases cause major troubles in shrimp farming (Lightner, 1996). Shrimp farming itself has got a significant effect on the environment such as loss of mangrove ecosystems, nutrient enrichment and eutrophication of coastal waters, development of antibiotic resistance in marine bacteria and accumulation of chemicals and toxicity to non-target species (Menasveta, 1997).

4. Bacterial disease, vibriosis

The reason of low level of aquaculture production has been due to a

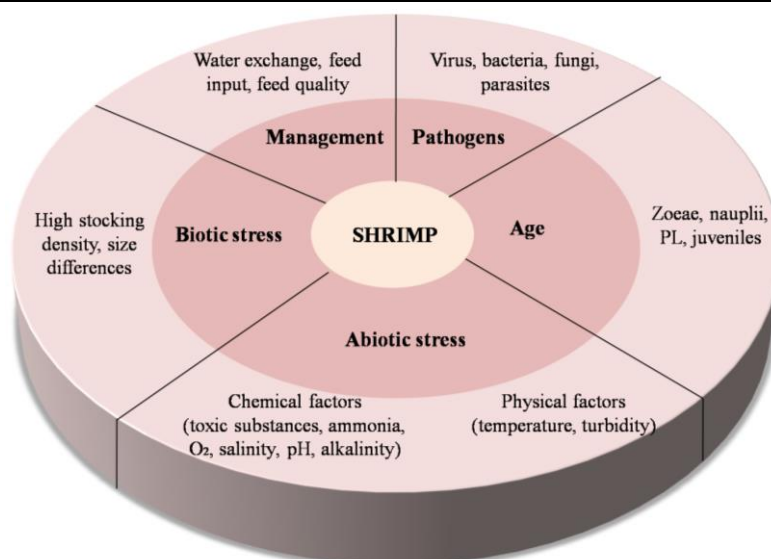


Figure 1: Factors influencing the susceptibility of shrimp to pathogens.

combination of both disease and pollution. While disease played the prominent role in declining aquaculture stocks, both factors caused massive mortality in the leading aquaculture countries. Among the groups of microorganisms that cause serious losses, the best known are bacteria because of the devastating economic effects they have on affected farms. Bacterial diseases mainly due to Vibriosis have been reported in Penaeid shrimp culture systems implicating several species of Vibrios which includes *Vibrio harveyi*, *V. splendidus*, *V. parahaemolyticus*, *V. alginolyticus*, *V. anguillarum*, *V. vulnificus*, *V. campbelli*, *V. fischeri*, *V. damsella*, *V. pelagicus*, *V. orientalis*, *V. ordalii*, *V. mediterrani*, *V. logei* etc. From the all above species *V. harveyi* is the main causative agent causing luminous vibriosis to larva in hatchery and pond cultivation (Won and Park, 2008). Being an important etiological agent, it causes mass mortalities in penaeid shrimp culture and leads to huge economic losses. They continue to cause chronic mortalities of up to 30% among *P. monodon* larvae, post larvae and adult under stressful conditions (Le Groumellec et al., 1996). In production unit it causes 100% losses at a time due to various virulence factors (Chythanya et al., 2002).

The genus *Vibrio* is a gram negative motile rod shaped gamma proteobacter.

The disease produced by *Vibrio* in shrimp culture is commonly called as vibriosis. The other names of bacterial vibriosis are luminescent vibriosis, penaeid bacterial septicaemia and red-leg disease (Aguirre-Guzmán et al., 2004). With the rapid developments in aquaculture particularly in Asia and South America, *V. harveyi* and related bacteria have become recognized as a serious cause of disease (Austin and Zhang, 2006). In many cases, *Vibrios* are opportunists causing disease when the host organism is immune-suppressed or otherwise physiologically stressed (Peddie and Wardle, 2005). Even though all crustaceans are suffered by this bacterium, most serious struggle was reported in Penaeid shrimp culture (Austin and Zhang, 2006).

The ill effects of adult animal due to vibriosis includes reddening body with red to brown gills, swimming lethargy and reduced feeding behaviours (Nash et al., 1992). Similarly infected PL showed empty gut and reduced motility and phototaxis. Based on the syndrome, they are expressed as localised and systemic vibriosis, oral and enteric vibriosis, septic hepatopancreatitis and appendage and cuticular vibriosis (Lightner, 1996). Luminescent *V. harveyi* appears to release exotoxins and may cause 80-100% mortality in *P. monodon* hatcheries (Harris, 1995). *Vibrio* species also cause red-leg disease

characterised by red colouration of the pleopods, periopods and gills in juvenile to adult shrimps. Shrimps suffering vibriosis may display localised lesions of the cuticle typical of bacterial shell disease, localised infections from puncture wounds, loss of limbs, cloudy musculature, localised infection of the gut or hepatopancreas and general septicemia (Lightner, 1993). Virulence factors associated with *V. harveyi* pathogenicity is due to the production of bacteriocin-like substance, phage induced haemolytic activity, extracellular products such as chitinases, cysteine protease, haemolysin, luciferase, metalloprotease, proteases, phospholipases, siderophores and proteinaceous exotoxins.

High loads of either *V. parahae-molyticus* or *V. harveyi* induced the rounding up and detachment of epithelial cells from the basal lamina of the mid gut trunk (MGT) and can cause high mortality in shrimp by eliminating two layers that protect the shrimp from infections: the epithelium and the peritrophic membrane. In addition, loss of the epithelium may affect the regulation of water and ion uptake into the body. It is well-known that there are significant differences between different *V. harveyi* isolates in terms of pathogenicity with some strains being highly virulent and others being non-pathogenic (Austin and Zhang, 2006). Even if many virulence factors have been already reported in *V. harveyi*, complete pathogenic mechanisms should be elucidated.

5. Quorum sensing in *V. harveyi*

The term ‘Quorum Sensing’ refers to the process of bacterial cell-to-cell communication. It is a population dependent phenomenon first characterized in the 1970’s in luminescent marine species of *Vibrio* (Nealson *et al.*, 1970). Through this mechanism bacteria coordinate gene expression in a density-dependent manner. It solely depends on the production, release and detection of chemical signal molecules called autoinducers (Miller and

Bassler, 2001). These molecules are constantly produced and received at a basal level by bacterial cells. With high population density, there is a surplus of signaling molecules in the environment. These signals diffuse back into the cell where they facilitate the regulation of gene expression (Hastings and Greenberg, 1999). The quorum sensing signal molecules are found to be involved in the regulation of various physiological processes such as the bioluminescence, biofilm formation, pigment production, toxin production, exopolysaccharide production, motility and virulence factor production in many Gram-negative bacteria including fish and shrimp pathogens like *V. harveyi* (Bruhn *et al.*, 2005; Kennedy *et al.*, 2006).

In each QS system, the autoinducer attaches to a gene that is known as the transcriptional activator and this attachment can lead to alterations in DNA and activation of virulence factors (Waters and Bassler, 2005). Gram-negative bacteria use N-acyl homoserine lactones (AHLs) as autoinducers, while Gram-positive bacteria use oligopeptides to communicate (Miller and Bassler, 2001). The most extensively investigated intercellular signaling molecules are the AHLs. Quorum sensing in *V. harveyi* is regulated via a multichannel phosphorylation / dephosphorylation cascade. This bacterium produces and responds to two autoinducers namely (i) *harveyi* autoinducer 1 (HAI-1) and (ii) autoinducer 2 (AI-2), which regulate the expression of bioluminescence and other virulence factors. Miller and Bassler (2001) have thoroughly studied the mechanism of QS in *V. harveyi*. HAI-1 is an AHL and its biosynthesis is catalysed by the luxM enzyme (Figure 2). AI-2 is a furanosyl borate diester and its biosynthesis is mediated by the luxS enzyme. Both HAI-1 and AI-2 are detected at the cell surface by the LuxN and LuxP-LuxQ receptor proteins respectively. In the absence of the signals, LuxN and LuxQ autophosphorylate and transfer phosphate to LuxO via LuxU. The phosphorylated luxO is an active repressor

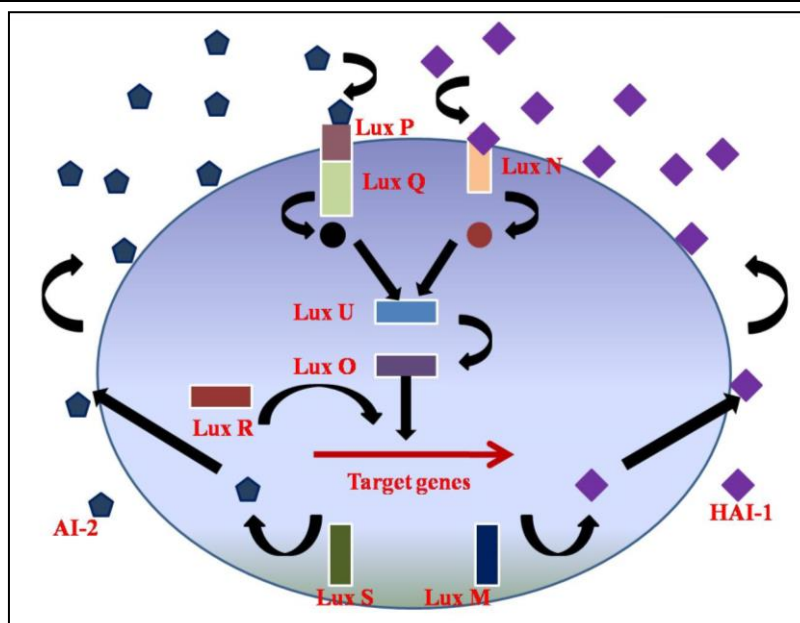


Figure 2: Mechanism of QS in *V. harveyi* (Miller and Bassler, 2001).

for the target genes. In the presence of the signal molecules, LuxN and LuxQ interact with their autoinducers and change from kinases to phosphatases that drain phosphate away from LuxO via LuxU. The dephosphorylated LuxO is inactive. Subsequently, transcription of the target genes is activated by LuxR. Recently, a third QS component, a *Vibrio cholerae*-like autoinducer CAI-1 was discovered and identified as (S)-3-hydroxytridecan-4-one in *V. Harveyi* (Henke and Bassler, 2004). Several investigations have been made to find the effect of *V. harveyi* quorum sensing on the production of the virulence factors like caseinase, gelatinase, lipase, hemolysin and phospholipase by determining their expression levels both *in vitro* and *in vivo* during infection of gnotobiotic brine shrimp (Natrah *et al.*, 2011; Darshanee *et al.*, 2011).

6. Antibiotics vs anti-vibrio probiotics

Traditionally antibiotics have been used in attempts to control bacterial disease in aquaculture. When antibiotics are used, the resistant strains can multiply rapidly because their (sensitive) competitors get removed. Moreover, horizontal exchange of resistant determinants can

occur between resistant bacteria and virulent pathogens that re-enter the culture facilities after the treatment. Thus, antibiotic-resistant strains of pathogenic *V. harveyi* can evolve rapidly. In view of the indiscriminate use of antibiotics in aquaculture, it cannot be surprising that many reports have mentioned multiple resistance of *V. harveyi* strains to several antibiotics (Table 1). From all this, it might be clear that the efficacy of antibiotics to treat luminescent vibriosis is very poor. The presence of residual antibiotics in commercialized aquaculture products constitutes another problem with respect to human health as this can lead to an alteration of the normal human gut microbiota and can also generate problems of allergy and toxicity (Cabello, 2006). Antibiotics residues in animals are also regarded as hazardous for exporting quality. In order to limit the use of antibiotics, many workers have been exploring the use of new bioactive compounds for controlling bacterial diseases of shrimp particularly that of caused by *V. harveyi*. Various solutions have been proposed such as the use of probiotics, immunostimulation, vaccination, specific pathogen-free (SPF) and specific pathogen-resistant (SPR) shrimp.

Table 1: Multiple antibiotic resistances in *V. harveyi* isolated from aquaculture facilities

Location	Antibiotic(s)	Multiple resistance	Reference
India	Cotrimoxazole, chloramphenicol, erythromycin and streptomycin	+	Karunasagar <i>et al.</i> , 1994
Java	Tetracyclin, ampicillin and other β -lactams	+	Teo <i>et al.</i> , 2002
Mexico	Ampicillin, amikacin, carbenicillin, cephalotin and oxytetracycline	+	Molina <i>et al.</i> , 2002
Philippines	Oxytetracycline, furazolidone, oxolinic acid and chloramphenicol	+	Tendencia and De La Peña, 2001
Philippines	Kanamycin, gentamycin, carbenicillin and ampicillin	+	Nakayama <i>et al.</i> , 2006
Taiwan	Nitrofurantoin, novobiocin and sulphonamide	+	Liu <i>et al.</i> , 1997
Thailand	Kanamycin and carbenicillin	+	Nakayama <i>et al.</i> , 2006

The addition of beneficial bacteria to exclude potential pathogens from shrimp larviculture has been suggested as early as 1991. Beneficial bacteria may enhance larval nutrition by supplying essential nutrients, improving digestion through essential enzymes, mediating direct uptake of dissolved organic material and producing substances which may inhibit the growth of opportunistic pathogens (Browdy, 1998). The potential negative consequences of using antibiotics in aquaculture have led to the use of non-pathogenic bacteria as probiotic control agents (Vaseeharan and Ramasamy, 2003). Probiotics are defined as “live microbial feed supplement which when consumed in adequate amounts confer a health benefit for the host”. As antibiotics become less popular for controlling the aquatic microflora in hatcheries the use of probiotics in aquaculture has become increasingly popular. The probionts commonly used for aquaculture are isolated from healthy larvae and adults. However, some probionts used for humans and terrestrial animals have also shown promise in aquaculture (Vine *et al.*, 2006). Probiotic use in aquaculture is practiced throughout the world and the results showed improved man-

agement of shrimp culture practices (Vaseeharan *et al.*, 2004). Probiotics in contrast to antibiotics can be a safer ecological alternative tool for sustainable aquaculture. In order to be considered as biological control agents in aquaculture, probiotics should be non-pathogenic and biochemically and physiologically well characterized. It should be genetically stable. They should be normal inhabitants of the host and able to survive and grow at the site of application while exerting their beneficial effect. Finally, they should maintain their viability and activity throughout the product manufacturing and storage.

Balcázar *et al.*, (2006) found that *V. alginolyticus* UTM102, *Bacillus subtilis* UTM126, *Roseobacter gallaeciensis* SLV03 and *Pseudomonas aestumarina* SLV22 are effective probiotics in preventing *V. parahaemolyticus* infection in shrimp culture. Feed conversion ratio, specific growth rate and final production were higher in shrimp receiving a probiotic mixture of five *Bacillus* species (*B. subtilis*, *B. licheniformis*, *B. polymyxa*, *B. laterosporus* and *B. circulans*) than in control shrimp which had received no probiotic (Ziaei *et al.*, 2006). *Bacillus* S11

bacterium provided protection in *P. monodon* shrimp when challenged with *V. harveyi*. After a 100 days feeding trial, shrimp in the treatment groups displayed 100% survival after challenge with *V. harveyi* whereas high mortality was observed in the control group (Rengpipat et al., 1998). Continuous addition of *Bacillus* sp. as probiotic to tanks containing black tiger shrimp for over 160 days decreased shrimp mortality caused by luminescent-pathogenic *Vibrio* (Moriarty, 1998).

Cell-free extract of *Bacillus subtilis* BT23 showed greater inhibitory effects against the growth of *V. harveyi*. Their probiotic effect was tested by exposing *P. monodon* larvae to *B. subtilis* BT23 before a challenge with *V. harveyi*. The results showed 90% decrease in accumulated mortality (Vaseeharan and Ramasamy, 2003). Decamp et al. (2008) reported some field data of the use of a. The addition of the commercial mixture of *Bacillus* strains in Thai and Brazilian hatchery water significantly improved the survival of *P. monodon* and *Litopenaeus vannamei* larvae. There have been reports that *Pseudomonas* species produce bioactive compounds with the ability to control vibrios such as *V. harveyi* and *V. parahaemolyticus* and that have no effect on the shrimp (Vijayan et al., 2006). The culture supernatant or culture filtrate of *Pseudomonas* sp. W3 contain secreted secondary metabolites (anti-vibrio) that inhibited the pathogenic bacteria responsible for shrimp luminous vibriosis disease. The active anti-vibrio compound produced by *Pseudomonas* sp. W3 is a small molecule with heat stable, pH resistant and mostly tolerant to a variety of enzymes such as lysozyme, protease, lipase and amylase (Rattana-chuai et al., 2010). A bioactive compound produced by *Pseudomonas* MCCB 102 and 103 that inhibited *V. harveyi* was identified as N-methyl-1-hydroxyphenazine, a phenazine antibiotic (Preetha et al., 2009). Cell free extracts of four *Bacillus* species isolated from Penaeid shrimp gut have shown the inhibitory

activity against luminous strain of *V. harveyi* (Ramesh and Umamaheswari, 2011).

Possible modes of action that have been mentioned in literature for probiotics include: (1) Production of inhibitory compounds, (2) Competition for nutrients, (3) Competition for adhesion sites in the gastrointestinal tract, (4) Enhancement of the immune response, (5) Production of essential nutrients such as vitamins and fatty acids and (6) Enzymatic contribution to digestion. A protocol for the development of probiotics as biocontrol agents in aquaculture was proposed by Verschuere et al. (2000) and later by Vine et al. (2006). It involves the following major steps: (1) *In vitro* screening, (2) Identification, (3) Pathogenicity or toxicity test, (4) *In vivo* validation and (5) Cost-benefit analysis. In addition to this, bacteria that are able to improve the water quality by removing toxic inorganic nitrogen or by mineralizing organic matter are also considered as probiotics.

7. Plant based anti-vibrio

Antibacterial compounds from natural resources would be the alternative to overcome the resistance problem in most of the pathogens. Screening of antibacterial activity of medicinal plants is very important since vast number of medicinal plants have been used for centuries as remedies for human and animal diseases. Much interest is now directed towards the vast untapped source of plant-based antimicrobials, many of which reduce the side effects of synthetic antimicrobials. Medicinal plant extracts have been used for centuries as remedies for animal diseases because they contain components of therapeutic value. Various chemotherapeutic agents isolated from plants have proved effective against drug-resistant bacteria. The role of plants in the discovery of drugs has increased notably in recent years due to a substantial improvement in biological screening methods. Unfortunately, the chemical nature of phyto-compounds present in many plants are still

has to be elucidated. Though numerous studies have reported the basic bioactive nature of various herbals, only a small percentage of that have been examined and explored thoroughly for their bioactive potential. Many terrestrial and coastal plants and marine seaweeds were reported to have promising antibacterial (anti-vibrio) activity against aquaculture pathogens including *V. harveyi*. Three mangrove species (*Avicennia marina*, *Bruguiera cylindrica* and *Acanthus ilicifolius*) collected from the coast was extracted in methanol and tested for different range of biological activities including antimicrobial activity against five species of fish and shrimp *Vibrio* pathogens (Manilal et al., 2009).

8. Anti-quorum sensing (quorum quenching)

Strategies to interfere with quorum sensing provide new avenues to combat bacterial diseases in humans, animals and plants. These strategies are termed as quorum quenching which targets different components of bacterial quorum-sensing communication systems and disintegrates quorum-sensing-dependent bacterial attacks. Due to the increase in antibiotic resistance, disruption of QS could lead to new pharmaceuticals and it can significantly decrease the virulence factor production in bacteria without interfering their growth. Hence, the disruption of quorum sensing (quorum quenching) has been suggested as a new anti-infective strategy in aquaculture and several techniques that could be used to disrupt quorum sensing have been investigated (Dong and Zhang, 2005). This can be done by one of the three methods: degradation of the enzyme that produces autoinducers, degradation of the autoinducers or degradation of the gene that the autoinducer attaches to and by autoinducers analog (Roche et al., 2004). Acylases and lactonases are two kinds of AHL-degrading enzymes also known as quorum sensing inhibitors (QSI) which have been identi-

fied (Figure 3). Lactonases block signal reception by removing the lactone ring from the AHL molecule resulting in just the acylhomoserine. Acylases detach the nitrogen bond to form a fatty acid chain and homoserine lactone. By changing the structure of the AHL molecules, lactonases and acylases keep AHLs from attaching to the transcriptional activator (Czajkowski and Jafra, 2008). Several AHL-degrading enzymes identified in various bacteria have the potential to be used as quorum quenchers. The first application of autoinducer quenching for the purpose of disease control involves *aiiA* [autoinducer inactivation (*aiiA*)], a *Bacillus* gene encoding AHL lactonase, which inactivates AHL by hydrolyzing its lactone bond (Dong et al., 2001). AHL degradation protects aquatic animals from infection, hence AHL-degrading *Bacillus* sp. might be interesting novel biocontrol strains for use in aquaculture. Similarly one of the first AHL acylases identified was *AiiD* from *Ralstonia eutropha* (Lin et al., 2003). The homologs of *aiiA* were later found in the closely related *Bacillus* species including *B. subtilis*, *B. cereus*, *B. mycoides* and many subspecies of *B. thuringiensis* (Dong et al., 2002; Lee et al., 2002; Pan et al., 2008; Uroz et al., 2003).

Medicinal plants contain large varieties of chemical substances with important therapeutic properties that can be effectively utilized in the treatment of animal diseases like Vibriosis caused by luminous *Vibrio* pathogens. Because of their history of medicinal properties, many folk medicinal plants have been screened for anti-quorum sensing activities. Among all the possibilities to inhibit QS activity, the use of anti-QS (AHL analogue) compounds may be of great interest to avoid bacterial infections. Therefore, screening of anti-QS compounds or QS inhibitors (QSI) from natural resources have been used for centuries as remedies for various diseases. QSI compounds have been identified from a wide range of natural resources particularly medicinal plants, edible vegetables and fruits, marine sponges

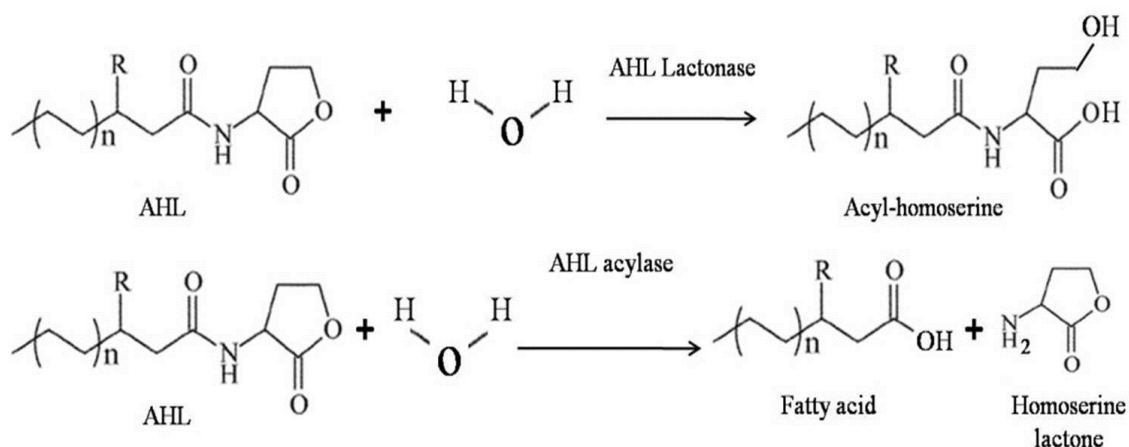


Figure 3: Mode of action of AHL lactonase and AHL acylase.

and seaweeds (Kim *et al.*, 2007; Skinder-soe *et al.*, 2008; Daglia *et al.*, 2010; Musthafa *et al.*, 2010).

The QS interference from novel sources may also be an important as the antibacterial effects. Anti-QS agents were first characterized in the red marine alga *D. pulchura* (Manefield *et al.*, 1999). This alga was investigated for its anti-fouling properties and was found to contain halogenated furanones which block AHLs via competitive inhibition and destabilization of LuxR (Manefield *et al.*, 2002). *Delisea* furanones have been shown to reduce light emission in *Vibrio* species (Givskov *et al.*, 1996), inhibit pigment production in *C. violaceum* (Martinelli *et al.*, 2004) and attenuate exoenzyme production and swarming motility in *Serratia liquefaciens* (Rasmussen *et al.*, 2000). The quorum sensing-disrupting natural furanone, (5Z)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone was found to block autoinducer 2 quorum-sensing in *V. harveyi* in a concentration-dependent way (Defoirdt *et al.*, 2006). Persson *et al.*, (2005) reported that toluene extracts of garlic contained several compounds with varying levels of QSI against Gram-negative transcriptional regulators Lux R or Lux R. Sergey *et al.*, (2011) have evaluated 78 natural products from chemical libraries containing compounds from marine organisms (Sponges, algae, fungi and cyanobacteria) and terrestrial plants were screened for the inhibition of bacterial QS using a reporter strain

C. violaceum CV017. Various fruits and herbs were shown to possess anti-QS activity in a *C. violaceum* biomonitor strain and on the swarming motility of *E. coli* and *P. aeruginosa* (Vattem *et al.*, 2007).

The subsequent discovery of compounds that inhibit cell-to-cell communication, dubbed anti-quorum sensing (anti-QS) agents could provide a novel method of combating infection. It is possible that several terrestrial plants also produce quorum signal mimics capable of controlling bacterial quorum sensing (Gao *et al.*, 2003). Even bacteria themselves produce QSI substances (Nithya *et al.*, 2010). Spices such as garlic, ginger and turmeric have been reported for their QSI potential (Vattem *et al.*, 2007). Similarly, the essential oils of cinnamon (Niu *et al.*, 2006) and clove (Khan *et al.*, 2009) are also known to possess QSI potentials. Acyl-homo serine lactone analogs and other quorum sensing inhibitors (QSI) have been investigated to determine their ability to prevent expression of quorum sensing controlled genes. The complex of signalling molecules (AHLs) and receptor proteins trigger the expression of specific genes responsible for bioluminescence in *V. harveyi* (LuxM/N). Hence the disintegration of signals with receptor by plant derived QSI prevents the bioluminescence and other virulence factors in *V. harveyi*. Several compounds have been identified that have the ability to interfere with QS-mediated gene expression through com-

petitive inhibition thus reducing biofilm thickness (Hentzer *et al.*, 2002).

9. Perspectives

The oceans cover more than 70% of the earth's surface and they are a promising source of novel pharmacologically active compounds. Although macroorganisms of the ocean have proved to be good sources of novel bioactive metabolites, large-scale productions of these bioactive metabolites have been difficult. Microorganisms isolated from marine sources have been reported to produce anti-bacterial, anti-fungal, anti-viral and anti-tumor substances. Several studies have suggested that such marine bacteria can be used as bio-control to combat epizootics in aquaculture systems. During the past two decades, the use of probiotics as an alternative to antibiotics has shown to be promising in aquaculture. Data about the impact of quorum sensing on virulence of aquatic pathogens are still lacking. Few reports that deal with disruption of quorum sensing of aquatic pathogens indicate that this new approach has potential in fighting infections in aquaculture. The furanones reported earlier as quorum quenchers (QS inhibitors) are toxic and chemically synthetic (non-degradable). Consequently the invention of non-toxic, broad spectrum QS inhibitors is needed for its successful exploitation against bacterial infections due to their drug resistance.

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Promiscuous Rhizobia: A Potential Tool to Enhance Agricultural Crops Productivity

Ikbal¹, Prasad Minakshi^{1*}, Basanti Brar¹, Upendra Pradeep Lambe¹, Manimegalai Jyothi¹, Koushlesh Ranjan², Deepika³, Virendra Sikka⁴ and Gaya Prasad⁵

¹Department of Animal Biotechnology, LUVAS, Hisar, Haryana, 125004, India;

²Department of Veterinary Physiology and Biochemistry, SVPUAT, Meerut, 250110, Uttar Pradesh, India; ³Department of Botany and Plant physiology, CCSHAU Hisar, 125004, Haryana, India; ⁴Department of Molecular Biology, Biotechnology & Bioinformatics, CCSHAU Hisar, 125004, Haryana, India; ⁵SVPUAT, Meerut, 250110, Uttar Pradesh, India; *Correspondence: minakshi.abt@gmail.com / minakshi.abt@luvas.edu.in; Tel: +91 9992923330

Abstract: *Rhizobium*-legume symbiosis is a complex and regulated association between plant and bacteria. This symbiosis is under the coordinated and tight regulation of several species specific (symbiosis related) genes of bacterium and respective host plant. Thus, rhizobia require action of several classes of specific genes for the formation of an effective symbiosis and dictate the host range. Other *nod* genes mediate the ‘decoration’ of the core signaling compounds with various substituents and make them host-specific. But, there are some reports that highlight that the rhizobia can infect non-legume plants. The signaling compounds are responsible for the effective symbiosis; however, there are several other factors which influence symbiosis and needs to be discovered. Certain modifications in the signaling molecules can cause changes in legume host range. Genetic exchange and rearrangement among heterologous *Rhizobium* spp. leading to broadening of host range and become promiscuous. Such type of rhizobia having broad host range and could be beneficial for the agricultural practices; because, choosing the correct inoculant group for a particular legume host is difficult for effective nodulation. Most of the commercially available strains are known to have a very narrow host range. Promiscuous *Rhizobium* strains for greater symbiotic association and ability to infect across strict host specificity would be of greater importance for the farming community. Farmers can enhance Biological Nitrogen Fixation by inoculating such correct rhizobia to their legume crops. The potential of this system is appealing because the whole world is seeking to adopt the organic farming. This could provide an alternate method to improve the soil fertility and could boost the agricultural sustainability.

Keywords: Biofertilizer; nitrogen fixation; promiscuous; *Rhizobium*

1. Introduction

Biological nitrogen fixation occurs mainly through symbiotic association of plants with N₂-fixing microorganisms (Shiferaw *et al.*, 2004). BNF supply nitrogen more than 2x10¹³ g/year to the world agriculture system (Falkowski, 1997). It is one of the most economically

system ever studied, involves bacteria (*Rhizobium*) and legume plants. The establishment of symbiosis involves several signaling molecules exchange between bacteria and host plant. These molecules are regulated by several *nod* genes and work in coordinated manner (Cohn *et al.*, 1998; Long, 1996). In a successful symbiosis rhizobia colonize on roots of host

plants and elicit the nodule formation where they colonize and differentiate into non-dividing endocellular symbionts. These symbionts convert atmospheric dinitrogen into NH_3 through the induction of the nitrogenase complex (Patriarca *et al.*, 2002). *Rhizobium* species have been successfully used worldwide as a bio-inoculant leading to effective establishment of nitrogen fixing symbiosis with leguminous crop plants (Miller *et al.*, 2007). Nitrogen applied as fertilizers usually provides high yields to plants. Therefore efficient monitoring of biological nitrogen fixation and status of chemical fertilizers are essential to balance the yield of crops and need to minimize environmental pollution, especially water and soil quality (Jaynes *et al.*, 2001). The role of BNF, especially in legumes, is well established and documented but Legume-*Rhizobium* symbiosis is not so extensively studied the system of nitrogen-fixation. Soil containing adequate and diverse communities (Figure 1) of rhizobia and become less effective at nitrogen fixing.

The application of sufficiently high numbers of improved inoculant strains can successfully compete with established soil rhizobia and replace them (Figure 2). The aim is to increase, the percentage of crops that are inoculated in terms of biomass yield and extra nitrogen in the soil. Improved *Rhizobium* strains for greater symbiotic association and ability to infect across strict host specificity would be of greater importance for the farming community. Farmers can enhance BNF by inoculating such correct rhizobia to their legume crops. Such promiscuous *Rhizobium* strains with improved efficiency to fix nitrogen would acts as a single inoculum for all the legumes and may add higher amount of nitrogen per unit area.

2. Classification of biofertilizers as per host specificity

Biofertiliser are the low cost source of plant nutrients, eco-friendly and have supplementary role with chemical fertiliz-

ers. The Bio-fertilizers are bacteria, algae and fungi and may broadly be classified into two categories viz. Nitrogen fixing like *Rhizobium*, *Azotobacter*, *Azospirillum*, *Acetobacter*, Blue Green Algae and Azola and Phosphorous solubilisers/mobilisers like PSM and Mycorrhizae (Figure 3). Rhizobia and legumes establish a mutualistic symbiosis. Host specificity is an important characteristic of symbiosis, where specific species of rhizobia forms nodules on defined legumes (Ampomah *et al.*, 2008). Rhizobia currently consist of 61 species belonging to 13 different genera, namely *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Azorhizobium*, *Allorhizobium*, *Sinorhizobium*, *Methylobacterium*, *Cupriavidus*, *Burkholdera*, *Devosia*, *Ochrobactrum*, *Herbaspirillum* and *Phyllobacterium*. Some Rhizobia have a narrow host range and form nodules with specific legume. For example *Azorhizobium caulinodans*, *Sinorhizobium saheli* and *sesbaniae* biovar of *Sinorhizobium teranga* nodulate only *Sesbania rostrata* (Boivin *et al.*, 1997) and *Rhizobium galegae* is the only symbiont of *Galega officinalis* and *Galega orientalis* (Lindstrom, 1989). In contrast some rhizobia are capable to infect a spectrum of legumes as they have broad host range (various degree of promiscuity). For example, *Sinorhizobium* sp. NGR234 and closely related *Sinorhizobium fredii* USDA257 nodulate at least 112 and 77 legumes from two different tribes, respectively (Pueppke and Broughton, 1999).

3. Symbiotic infection is a regulated pathway

Symbiosis is a developmental process driven by bacteria but ultimately under the control of host plant (Murray, 2011). The successful establishment of infection requires several factors such as nod factors and plant exudates (flavonoids). These flavonoids activate different kinds of nitrogen fixing genes and the interaction takes place between bacteria and

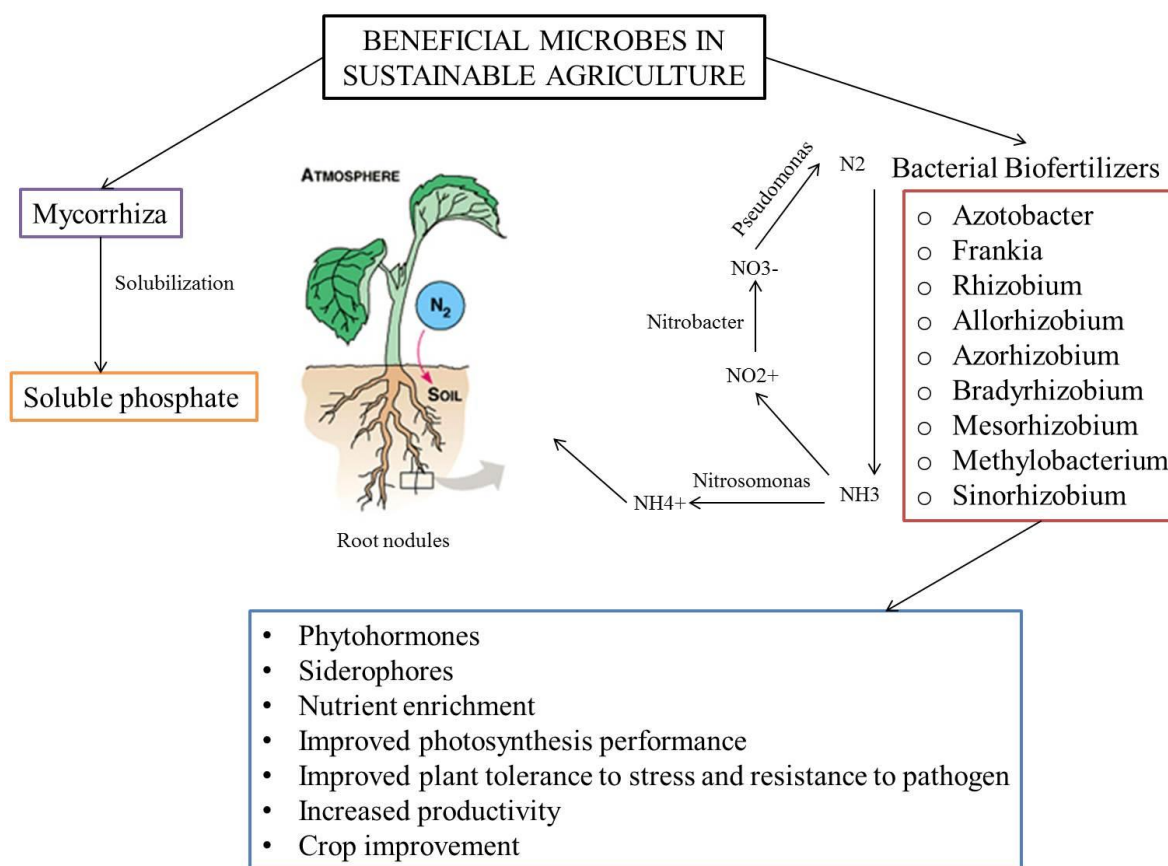


Figure 1: Diverse communities of microorganisms found in soil.

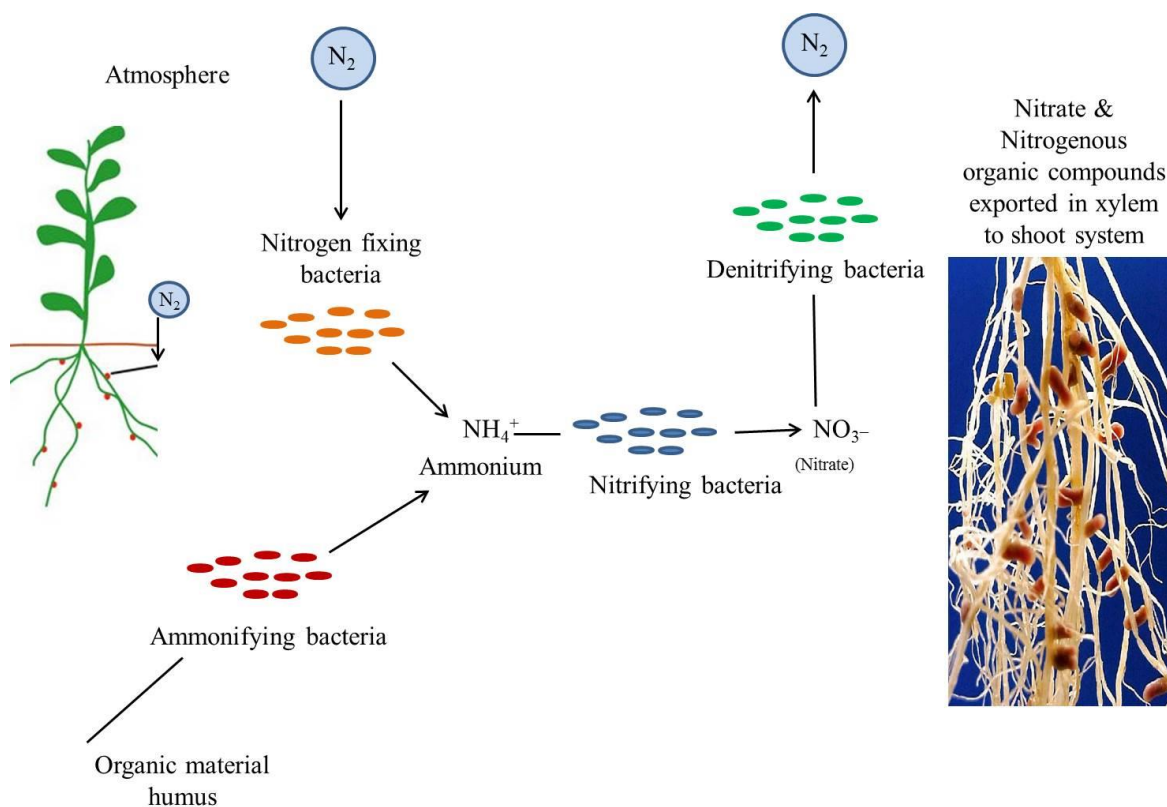


Figure 2: Atmospheric nitrogen fixation by microorganisms.

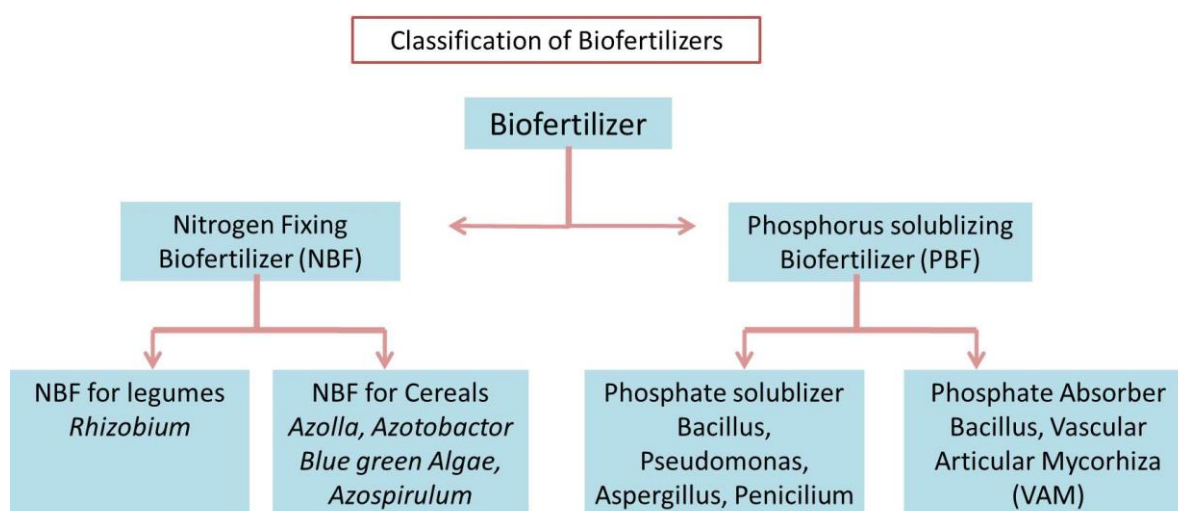


Figure 3: Classification of biofertilizers based on microorganisms.

plants. In positive interaction rhizobia moves towards the localized sites of plant root and followed by formation of infection pocket (Barbour *et al.*, 1991; Brewin, 2004). A bacterial colony established in this infection pocket and become a new organ called nodule (Fournier *et al.*, 2008 and Oldroyd and Downie, 2004).

These pre infection responses ready the plant for infection by rhizobia. However some β -rhizobia use an alternative pathway to initiate symbioses in some legumes, where a purine derivative plays a key role in triggering nodule formation. The universality of the nod factor paradigm was recently overturned by some bacteria that elicit root and stem nodules on a particular group of plants lack the canonical *nodABC* genes required for the synthesis of the Nod factor (Giraud *et al.*, 2007). This indicates that a group of rhizobia uses a NF-independent strategy to enter into symbiosis. Madsen *et al.*, (2010) found that *snf1/nfr1/nfr5* triple mutants allowed rhizobia to invade through an “intercellular” route (crack entry) but in this case rhizobial infection was not accompanied by the formation of infection threads within root hairs. This finding suggests that alternative pathway may facilitate the entry of the bacterium in to the roots of diverse legumes.

4. Specificity of symbiotic infection between legume plant species and rhizobia

Work on molecular basis of host specificity began at the end of the last century. Experimental evidence suggests that the progression of invasive rhizobia towards nodule primordial is challenged at various steps. The host range is determined at early stages of the plant-bacterium interaction. During initial phases of nodulation (bacterial entry), molecular signals are given by flavonoids and Nod factors (Martinez *et al.*, 1988). In this process, NodD proteins are the chief interlocutors of molecular traffic in the rhizosphere (Perret *et al.*, 2000). NodD shows specificity to certain flavonoid secreted by plants (Figure 4). Therefore, NodD takes part in determining host specificity (Miller *et al.*, 2007). Although some host plants and rhizobia have the ability to enter into symbiosis with more than one companion, only certain combination of symbionts results in the formation of nitrogen fixing nodules. Several other studies have also shown that the length of the oligosaccheride backbone of LCOs determine the host specificity of nodulation (Bec-Ferte *et al.*, 1994; Felle *et al.*, 1995; Heidstra *et al.*, 1994; Stokermans *et al.*, 1995). These results demonstrate that *nodC* contributes to the host specificity of *rhizobium*.

The amount of Nod factors released by rhizobia also play important role in determining the host range. For instance,

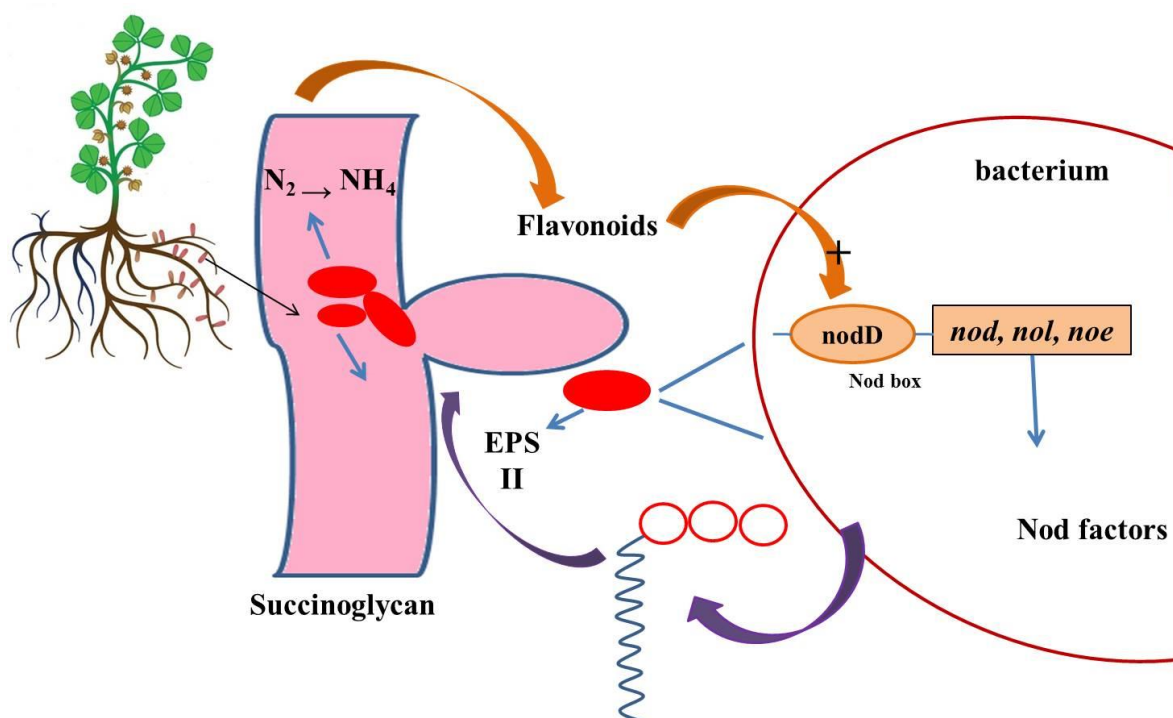


Figure 4: Symbiotic interaction between legume plant species and rhizobia.

introduction of strain NGR234 *nodD1* into *R. Meliloti* increases Nod factor production by about two-fold and permits the nodulation of *V. Unguiculata*, a non-host (Relic *et al.*, 1994). In *S. Meliloti* these NodD proteins respond to different groups of flavonoids, suggesting that NodD redundancy allows the bacterium to infect multiple hosts secreting a wide range of flavonoids (Maillet *et al.*, 1990). To demonstrate that *nod* is a key determinant of host specificity, Melicent *et al.* (2006) expressed *nod* genes from different species of rhizobia in a strain of *S. Meliloti* which was lacking endogenous *nodD* activity. They observed that *nod* gene expression was initiated in response to distinct set of flavonoid inducers. Furthermore, data from several researches suggest that *nodD* controls the response of rhizobia to flavonoids in species-specific manner (Hovath *et al.*, 1987) Herman *et al.* (1989) found that node product is the main factor that distinguish the host range for symbiosis. Hybrid *nodE* genes, which consist of a 5' part *Rhizobium leguminosarum* *nodE* genes and a 3' part of the *Rhizobium trifolii* gene, were constructed. From the properties of these

hybrid genes it was concluded that a central region determine different host ranges. Louise *et al.* (2002) have mutagenised *Rhizobium* strains with transposon Tn5 to determine if additional negatively-acting traits exist that can alleviate cultivar-specific nodulation failure. They reported two new mutants, proficiently nodulate cv. Woogenellup. They suggested that simple gene to gene interaction is not sufficient for symbiosis but there are at least two independent mechanisms which mediated the cultivar-specificity. Although much information is available on the influence of Nod factors on the host range yet no strict correlation can be drawn the types of LCOs produced by rhizobia and host plants.

5. Molecular factors control the symbiosis

The nitrogen fixation and nodulation by *Rhizobium* strains is controlled at various levels by certain factors (Hooykaas *et al.*, 1981). The importance of each individual step would depend on the specific legume–*Rhizobium* combination and Nod factors (NF) in the rhizosphere (Figure 5).

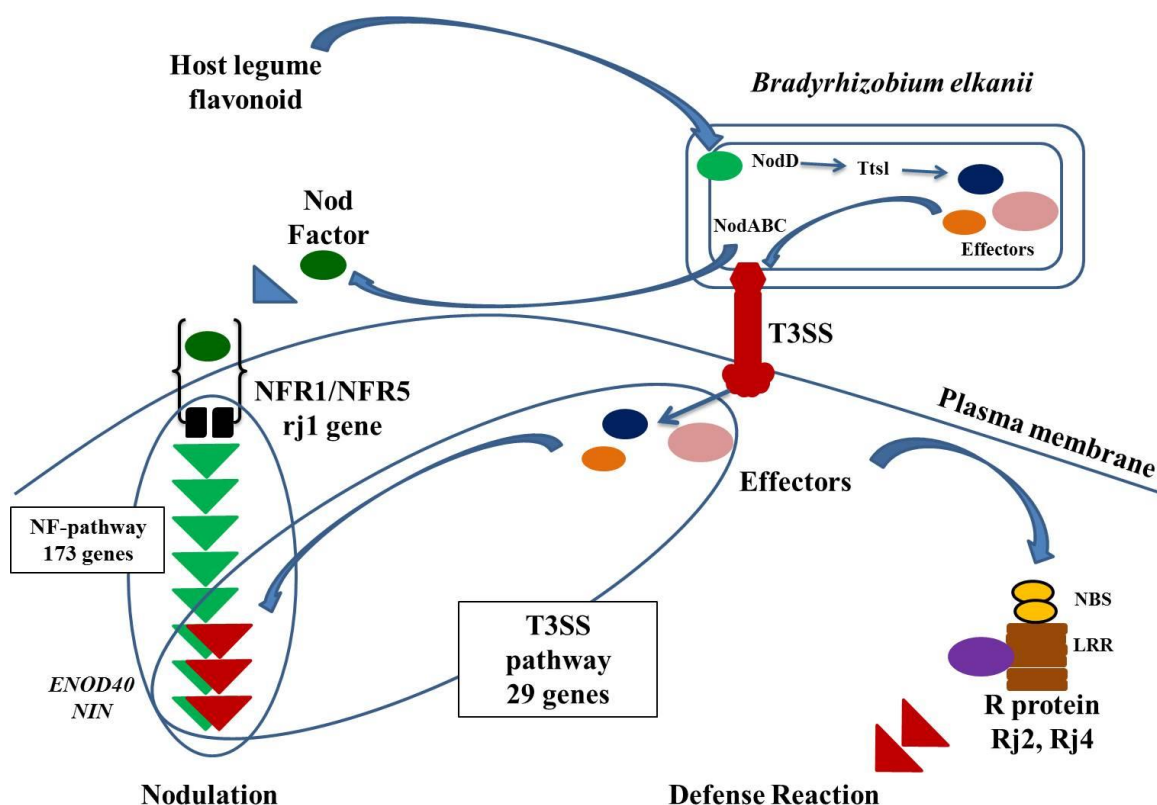


Figure 5: Molecular factors which are involved in symbiosis (Okazaki *et al.*, 2013).

Bacterial Nod factors function as a key to open the door of its host (Perret *et al.*, 2000), and there is a high degree of stringency for chemical structure of Nod factor that determine whether the host allows bacterial invasion to proceed. Nod factor elicits significant changes in the expression of host gene (D'Haeze and Holster, 2002; Oldroyd and Dowine, 2008). Nod factors are complex signaling molecules secreted from bacteria as a cocktail of β 1-4-linked N acetyl D-glucosamine (GlcNAc) trimers, tetramers or pentamers (D'Haeze and Holster, 2002). The host-rhizobia co-evolution involved modifications of Nod factor structure such as replacement of fucosyl, which made the interaction more specific and increased affinity between partners (Mario *et al.*, 2006).

All the rhizobial species have common nod genes (*nod A*, *B*, and *C*), which are capable of cross species complementation and responsible for the synthesis of nod factor backbone. These genes confer specificity for nodulation of

a particular host and are involved in various modification of the chitin backbone (Gibson *et al.*, 2008). The nodulation gene expression is activated when bacteria perceive flavonoids that are secreted by plant roots (Perret *et al.*, 2000). *nodD*, gene is central to the regulation of nod box which activates other *nod* gene expression (Loh and Stacey, 2003). It induces the transcription of nodulation genes involved in the synthesis of nod factors (Capela *et al.*, 2005 and Peck *et al.*, 2006). In response of isoflavone signals which are produced by plants *NodVW*, positively regulate *nod* genes, thus these are thought to activate transcription via a series of phosphorylation steps. Mutation in either of these two genes results in the complete loss of nodulation activity in certain plant hosts. The proposed role of *nodV* as a sensor of plants signals adds another point of complexity of *nod* gene regulation. In response of plant signal, phosphorylation of *NodV* takes place which subsequently activate *NodW* via the transfer of phos-

phoryl group to an aspartate residue in its receiver domain. The nodW play a key role in the regulation of nod gene expression and in the ability of *B. Japonicum* to infect the host plant (Sanjuan *et al.*, 1994). The phosphorylation of NodV and NodW is essential not only for nod gene expression but also for the nodulation. Nod factors permit rhizobia to enter their hosts and certain additional factors probably act within plant (D'Haeze *et al.*, 1998). Thus a number of physiological responses to nod factor are observed when rhizobium applied to the plant roots (Gibson *et al.*, 2008).

Said *et al.*, (1998) have found that *nolO* factor is the principal host range determinant. They have mobilized large fragments of the symbiotic plasmid of *Rhizobium spp.* NGR234 into heterologous rhizobia. The trans-conjugants nodulate *V. unguiculata* at low frequency and extended the host range. They have confirmed that the conjugation of *nolO* into *Rhizobium fredii* extends the host range of the recipient to the non-hosts. Nod factors are essential to the nodulation and their modification contributes to host specificity, thus these signaling molecules probably one of the several elements specifying host range. It should also be noted that although *nodFE* mutants of *Rhizobium melilotii* secrete nod factors in which C16 unsaturated fatty acids are replaced by vaccenic acid, the mutant still form nodules on various Medicago cultivars (Ardourel *et al.*, 1994). Several other examples contradict the dogma that Nod factors determine host specificity. Despite the fact that predominant nod factors secreted by *R. Leguminosarum* bv. *trifolii* and *R. Leguminosarum* bv. *viciae* are identical yet these two bacteria have distinct host ranges (Orgambide *et al.*, 1995). In contrast, two rhizobia that secrete different nod factor may nodulate the same plant: *R. Tropici* and *R. etli* produce different nod factors but both effectively nodulate *Phaseolus vulgaris* (Poupot *et al.*, 1995). It would thus seem that nod factors in absolute level are not only im-

portant for induction of different components of the nodulation pathway but also some bacterial cell surface components such as LPS, cyclic- β -glucans, EPS, capsular proteins and K antigens recognised by plants help to determine host specificity (Mathis *et al.*, 2005). These additional factors must have role in symbiotic development between rhizobia and plant.

6. Broadening of host range (promiscuity) of rhizobia

The host specificity concept has now almost defunct, because many overlapping host ranges have observed so the concept of host specificity has been challenged. A single legume *e.g.* Acacia, Glycine max or Leucaena can be associated with genetically dissimilar symbionts. Closely related rhizobia infect legumes from different tribes and distantly related rhizobia infect closely related legumes (Quesada *et al.*, 1997). The *Rhizobium sp.* strain NGR234 is good examples of this phenomenon. It has broad host range and nodulate legume species from 112 genera and the non-legume *Parasponia* (Pueppke and Broughton, 1999). Zhu *et al.*, (2002) characterized the rhizobia that nodulate legume species of the genus *Lespedeza* by analysing whole cell proteins, and cross-nodulation with selected legume species. They have observed that the strains isolated from *Sesbania spp.* and *Lespedeza spp.* represent a cross-nodulating group of bacteria. Hernandez-Lucas *et al.*, (1995) also found two strains of *R. etli* and three stains of *R. tropici* and tested on 43 legume species. Out of these 22 of the tested legume species were nodulated by three or more of these strains. These strains have broad host range and nodulate woody species also such as *Albizia lebbek*.

Setiyo Hadiwaluyo (2011) characterized forty one cross inoculating rhizobial isolates from Java and Sumatra and these isolates were used to inoculate soybean and mungbean plants. He found 19 isolates from Java and 15 isolates from

Sumatra were promiscuous. Beatrix *et al.*, (1987) have studied *nodD* gene from wide host range rhizobium strain MPIK3030 to verify the *nodD* function, as well as the host-range extension ability of *R. Meliloti*. The 2.9-kb *nodD* region was mobilized into *R. Meliloti* and trans-conjugants were tested for their nodulation phenotype on siratro and alfalfa. The double mutant *R. meliloti nodD*I12 PP659 carrying the MPIK3030 *nodD*I-region (pBH264), showed a clear restoration of nodulation on alfalfa and simultaneously extended the host range of the *R. meliloti* trans-conjugants to siratro. Similarly Transfer of *nodD*1 of NGR234 into *R. meliloti* results in host range extension to *M. atropurpureum* and *Vigna unguiculata*, whereas *R. meliloti nodD*1 is incapable of restoring the ability of an NGR234 *nodD*1 mutant to nodulate *M. atropurpureum* (Relic *et al.*, 1994). Conjugation of NGR234 *nodD*1 into *R. leguminosarum* bv. *Trifolii* extends its host-range to the non-legume *Parasponia andersonii* (NGR234 host) whereas *nodD*1 mutant of *R. trifolii* did not regain the ability to nodulate *Trifolium repens* when it was complimented with *nodD*1 of *R. meliloti* (Spaink *et al.*, 1987).

Promiscuity is not only the characteristics of the rhizobia, but some legumes also harbor diverse rhizobia (Perret *et al.*, 2000). Several plants such as *Phaseoleae* are nodulated by *R. leguminosarum* bv. *Phaseoli* as well as *Bradyrhizobium* and *Sinorhizobium* species (Gaultieri and Bis-seling, 2000). Arya K. Bal (1982) studied how a legume interacts with *Rhizobium* species of two different cross inoculation groups. They have compared physiology and morphology of root nodules induced by two *Rhizobium* species of different cross inoculation groups. They have found that *Rhizobium* sp. 127E15 promiscuously induce effective root nodules on pole bean. Similarly, Shantharam and Peter (1982) have shown that *R. phaseoli* 127K14 is capable of forming effective nodules on different legume. They have showed that *R. phaseoli* 127K14 nodu-

lates a host of different cross-inoculation group. Promiscuity is probably ancestral to restricted host range. In this support hypothesis comes from the observation that NGR234 and USDA257, both nodulate *Parasponia andersonii* (van Rhijn *et al.*, 1996). There are some other reports of promiscuous strains that have broad host range and nodulate soybean as well as many other legumes, including cowpea, pigeon pea and mungbean (Scholla and Elkan 1984; Stowers and Eaglesham 1984; Chamber and Iruthayathas 1988). In view of these reports it was concluded that promiscuity is widely dispersed in nature and not only associated with a particular bacterial or plant taxonomic group. In further studies, nodulation capacities of large collections of rhizobia have been evaluated by inoculation of numerous legumes.

7. Hydrolytic-cell wall degrading enzymes in rhizobial infection

In the development of the Rhizobium-legume symbiosis localized erosion of cellulosic plant wall is the central event through which the bacterial symbiont enter into host plant and establish a nitrogen fixing, intracellular endosymbiotic state. Previous studies found that rhizobia produce hydrolytic enzymes capable of degrading the cell wall polymers, but little is known about their molecular mechanism (Angle 1986). In considering the process of active penetration of plant cell wall by *Rhizobium* sp., McCoy (1932) was the first to investigate the involvement of hydrolytic enzymes. Ljunggren and Fahraeus (1961) gave the “polyglacturonase hypothesis” which describes the involvement of pectolytic enzymes at the site of nodule formation. The hypothesis in essence proposes a physical penetration of the root hair cell wall. Callaham and Torrey in 1981 gave the strongest evidence for the involvement of wall hydrolysis by *R. leguminosarum* bv. *trifolii* in white clover infection process. Baker *et al.* (1989) also found that many cells of *R.*

leguminosarum bv. *trifolii* attached to the root surface of white clover and produce pit erosions in epidermal wall that follow the penetration of the bacterium, suggesting that wall-degrading enzymes are involved in symbiosis. Vashishat *et al.*, (1985b) observed that *R. trifolii* strains were capable of producing hydrolytic enzymes like *pectinase*, *hemicellulase* and *cellulose* and these enzymes play important role in symbiosis. Considering these evidence Angle in 1986 tried to determine whether differences exists between fast and slow growing soyabean rhizobia to produce *pectinase* and proteolytic enzymes. It was proposed that wide spread production of proteolytic enzymes indicates indirect evidences for their involvement in the invasion of host. Al-Mallah *et al.* (1990) pre-treated clover roots with an enzyme mixture of 1% (w/v) *cellulase* and 0.1% *pectolase* before inoculating clover with *R. trifolii*. Increase in nodulation indicated the role of *cellulase* and *pectinase* in nodulation process of clover.

In 1992, Pedro *et al.*, verify the production of *R. leguminosarum* bv. *trifolii* enzymes that deteriorate polygalacturonate and carboxymethyl cellulose (CMC) as model substrates of plant cell wall polymers. Similarly Mateos *et al.* (1992) reported the production of enzymes from *R. leguminosarum* bv. *trifolii* that degrade carboxymethyl cellulose and polypectate substrates. Their studies shows that *R. leguminosarum* bv. *trifolii* produces multiple enzymes that cleave glycosidic bonds in the plant cell wall. Mateos *et al.*, (2001) also found that *cellulase* (Cel2) enzyme is important for symbiotic development because rhizobial symbionts require its activity to breach the host barrier to establish nitrogen fixing association with legumes. Robledo *et al.* (2008) have purified cell bound *cellulase* (Cel2) isozymes and analysed its symbiotic function by reverse genetics and plant microscopy approaches. These results provide compelling evidence that this enzyme could erode the tip of root

hair wall of the host and making a localized hole of sufficient size to allow rhizobial cell penetration. This leads to develop more nodules for successful nitrogen-fixation.

Hussain *et al.* (1995) studied the involvement of hydrolytic enzymes in the nodulation of berseem (*Trifolium alexandrinum*). They selected a single mutant hrt20m7 in wild type strain hrt20 of *R. trifolii* by screening for reduction in activity of degradative enzymes, the relative activities shown by the mutant for *pectinases* and *cellulase* were 33 and 4 percent, respectively of wild type strain. It was observed that mutants unlike its parent failed to nodulate clover seedlings. Aggarwal *et al.* (2000) found rhizobia behaving as super nodulating rhizobia. They suggest *cellulases* over *pectinases* in the process of symbiotic infection of berseem by *R. leguminosarum* bv. *trifolii*. They derived rhizobia mutants showing better growth on CMC and /or pectin *i.e.* behaving as super-nodulating rhizobia. Emtiazi *et al.* (2007) also studied the *cellulase* activities in nitrogen fixing *Paenibacillus* isolated from nitrogen free media. The *cellulase* positive *Paenibacillus* were selected by reduction of congored color on CMC medium. They have observed that nitrogen fixing strains with *cellulase* activities grow well on nitrogen free media with sucrose or manitol as the only sources of carbon. They have concluded that most plant associated microorganism might have *cellulase* activities for adoption or establishment of a plant microbe interaction. Egamberdieva *et al.* (2010) determined the bacterial *cellulase* activity on media containing the substrate carboxy-methylcellulose. They have found that *cellulase* producing bacteria were significantly increase nodule numbers and nitrogen content of the plants. Fouts *et al.* (2008) studied the complete genome sequence of the nitrogen fixing broad host range endophyte *Klebsiella pneumonia* 342. They have found the gene related to carbohydrates, including pectins and cellulosic compound degradation are essen-

tial for Kp342 to form endophytic associations. So, it was concluded that hydrolytic enzyme activity in the form of *cellulase* and *pectinase* is an essential property of *Rhizobium* for infection of white clover during symbiosis.

8. Improvement in symbiotic efficiency of rhizobia

The rhizobium-legume symbiosis accounts for a significant proportion of nitrogen available to leguminous plants. Thus there is a need to improve rhizobia to increase their symbiotic efficiency and host range. The traditional method for obtaining *Rhizobium* strains with improved properties has been the selection of naturally occurring field isolates that best exhibit the trait desired (Figure 6). An alternative approach is to construct improved *Rhizobium* strains by genetic transfer of symbiotically favorable determinants. Genomic rearrangements have been reported to occur frequently in *R. leguminosarum phaseoli* (Flores et al., 1988, Garg et al., 1999).

Better nitrogen fixation may be brought by manipulating both rhizobia and plant host by eventually creating an artificial rhizosphere. Schlaman et al., (1998) observed nodulation and the levels of nitrogen fixation can be significantly higher when plants are infected with rhizobia containing the hybrid gene *nodD604*, which activates the transcription of *nod* genes independent from flavonoids. For introduction of foreign DNA into bacterial species electroporation is a novel approach (Chassy et al., 1988). Garg et al. (1999) successfully carried out electro-transformation of *R. leguminosarum* with 15.1kb plasmid, pMP154 (Cmr), containing a *nodABC-lacZ* fusion by electroporation. Chitchanok et al. (2011) derived mutants from wild type *Rhizobium* sp. 6-1C1 through 0.8 and 1.0 kGy gamma radiation. They have observed that *Rhizobium meliloti nodH* gene mutants result in a change of host range. They infect vetch with this mutated strain but fail to nodulate their normal host, alfalfa.

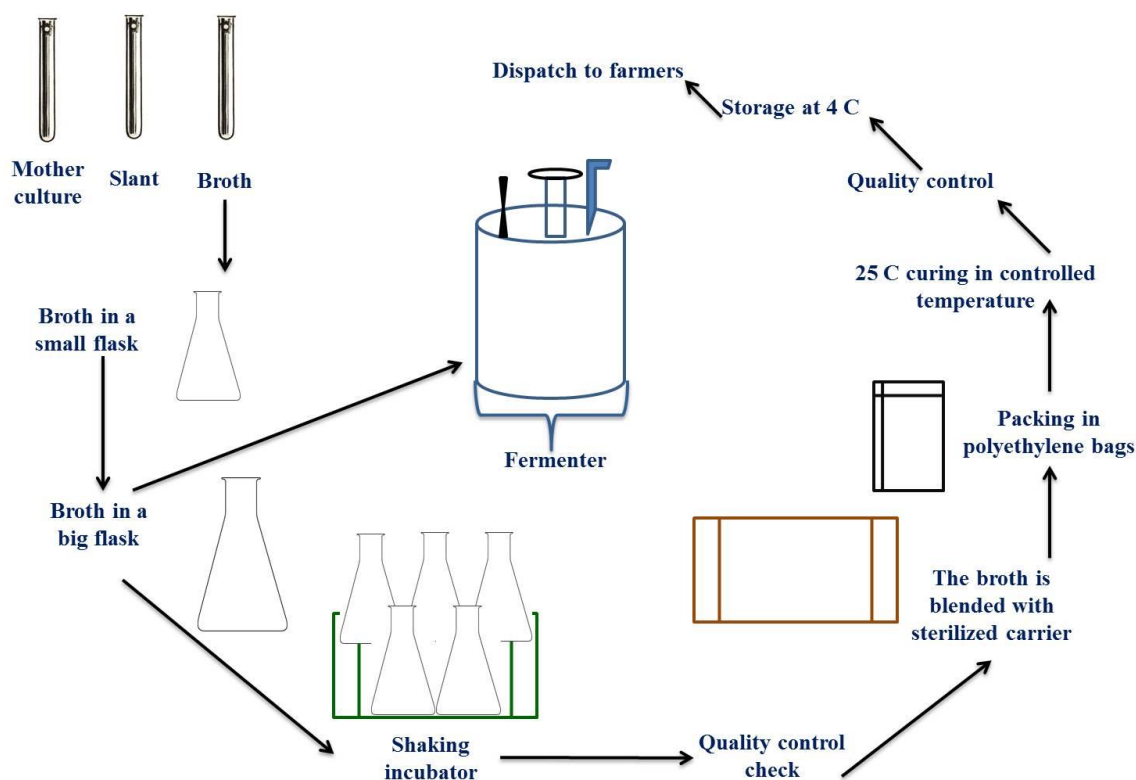


Figure 6: Improvement and large scale production of biofertilizers.

The *nodQ* on the other hand, are able to infect both alfalfa and vetch. Mostly mutations results in the alteration or extension of the host range (Faucher *et al.*, 1989 and Horvath *et al.*, 1986). In *R. leguminosarum* bv. *viciae* and bv. *trifolii*, the *nod* product is the main factor that distinguishes the host range for nodulation. In contrast to wild type, *R. leguminosarum* bv. *Trifolii nodEF* mutants nodulate white and red clover poorly but have acquired the ability to infect peas. When these *nodEF* mutants harbour the *node* gene of *R. leguminosarum* bv. *Viciae*, they have an extended host range to *vicia* and *lathyrus* species (Spaink *et al.*, 1989).

Transfer of *nodD1* gene of strain NGR234 to restricted host range rhizobia extend the nodulation capacity of the recipients to new hosts, including the non-legume *Parasponia andersonii* (Horvath *et al.*, 1987). Nonetheless, *nodD* gene represents a molecular interface between the bacterium and the plant. Plasmid transfer may increase nodulation or nitrogen fixation in *R. Leguminosarum* bv. *viciae* strains (DeJonj *et al.*, 1982), and there is one report of a plasmid loss that improves symbiotic properties in *Rhizobium loti* (Pankhurst *et al.*, 1986). In *R. meliloti*, a non-symbiotic plasmid enhances nodulation of the strains harboring it (Urban, J. 1988). Esperanza and Monica (1990) genetically modified *R. leguminosarum* bv. *phaseoli* CFN42, through transfer of a 225kb plasmid from typeII strain CFN299. They have observed that more nodules were obtained with the transconjugants on *P. vulgaris*. These strains also have a diminished competitive ability. Philippe *et al.*, (1996) introduced an IncP plasmid, pGMI149, carrying the main *R. meliloti* nodulation region into *R. tropici*. The *R. tropici* (pGMI149) transconjugants poorly nodulate *M. sativa*. When a second plasmid, of the IncQ group (compatible with pGMI149), carrying the *nodL* gene (pGMI1962) was introduced into *R. tropici* (pGMI149), a better nodulation was observed. They have also prepared NFs from *R. tropici*, *R. meliloti* (with

pMH682 for increasing NF production), and the *R. tropici* (pGMI149) (pGMI1962) hybrid strain and then tested the ability of these NFs to form nodules on alfalfa. NFs produced by the *R. tropici* hybrid strain were able to induce nodule formation. The presence of *R. meliloti* nodulation genes therefore enables *R. tropici* to produce new NFs that can induce nodule formation. Their results show that allelic variation of the common *nod-ABC* genes is a genetic mechanism that plays an important role in signaling variation and in the control of host range. They have also found that mutations in the regulatory genes *nodD1* and *nodD3* did not result in a detectable decrease in nodulation.

Falguni *et al.*, (2009) amplified 2.4 kb *fegA* gene (encoding ferrichrome receptor) along with its native promoter from *Bradyrhizobium japonicum* 61A152 and cloned in a broad host range plasmid vector pUCPM18. The plasmid construct pFJ was transferred by conjugation into *Rhizobium* sp. ST1 to give trans-conjugant ST1pFJ12. Inoculation of pigeon pea seedlings with trans-conjugant ST1pFJ12 led to a marked increase in plant growth parameters as compared to plants inoculated with the parent strain ST1, Nodule occupancy on pigeon pea plant when inoculated with the trans-conjugant was increased. Gene *fegA* not only supports the growth of the trans-formants rhizobia under iron limited laboratory conditions, but also increases its survivability under natural soil conditions, which led to higher nodulation on peanut plant. Yoshitake *et al.*, (2010) introduced *vktA* into *R. leguminosarum* cells and the strain with a remarkably high *catalase* activity was constructed. The *vktA* trans-formant was inoculated to the host plant *P. vulgaris* and the nodulation efficiency was evaluated. The nitrogen-fixing activity of nodules was increased 1.7 to 2.3 times as compared to the parent. Results show that the increase of *catalase* activity in rhizobial cells could be a valuable way to improve the nodulation and nitrogen-fixing ability

of nodules. Therefore, it was concluded that some genetic determinants of rhizobia involved in host range infectivity have been worked out leading to the extension of their infectivity but their effectivity in terms of *nitrogenase* expression in enlarged host is still not achieved. *Rhizobium* symbiosis with non-legume host clearly indicates the ability of this symbiont to nodulate legume as well as non-legume (Louise et al., 2002).

9. Perspective

Considerable research efforts have been made through development of promiscuous rhizobia for improving the efficiency of biological nitrogen fixation; because, this process has the potential to reduce our dependence on nitrogenous chemical fertilizers.

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Organic Farming and Halalan Toyyiban Foods: An Attempt to Relate Them

Quamrul Hasan^{1,2,*} and Zakirah Othman¹

¹Knowledge Science Research Lab., School of Technology Management and Logistics, College of Business, Universiti Utara Malaysia, 06010 Sintok, Kedah, Malaysia; ²Japan Halal Research Institute for Products and Services (JAHARI), Kobe, Japan; *Correspondence: quamrul@uum.edu.my

Abstract: Everyone wants to consume safe and healthy food. Also, the producers want to position their products according to the customers' demands. In the context of safe and healthy foods, among others, there are two different terminologies, 'Organic' and 'Halalan Toyyiban'. However, our understandings on these two terminologies are not clear enough especially when it comes to relate them. Therefore, this research work was undertaken to better understand the terminologies - organic and halalan toyyiban, and find out the relationship between them, if any. The research methodology involves both primary data by visiting an organic farm and face-to-face interviewing farmers, and secondary data. The findings might help the consumers in selecting the produce/product and business people in promoting their products. Research informants were farmer, volunteer, and intern at the Sri Lovely Farm, a government-certified organic farm at Sik, Kedah, Malaysia. The research reveals new insights on the relationship of characteristics of organic farming with halalan toyyiban. The three commonly found characteristics are: 1) quality; 2) healthy; 3) environmental friendly. Based on the findings, we are proposing a model on the relationship of organic farming with halalan toyyiban. This study is the first of its kind and undertaken as an exploratory research; therefore, further study should be conducted to obtain more understanding and knowledge on this subject.

Keywords: Environment; halal food; halalan toyyiban; organic farming; organic food

1. Introduction

In the last two decades, globalization has significantly advanced leading to not only technological and economic advancement but also in agriculture, food production, food safety and security. While efforts to establish trade rules led by the World Trade Organization and further progress in global free trade are advantageous to create new and mutually benefitted opportunities, such developments have highlighted several risks associated with agriculture and food, which were originally characterized by an unclear food chain (Huynen *et al.*, 2005).

Consumers are mainly concerned about health issues, protection of the environment and animal welfare besides food safety in terms of food processing methods, innovative food technologies, and presence of chemical substances in foods such as pesticides, toxins and food additives (Borin *et al.*, 2011; Hansen *et al.*, 2011; Stanton *et al.*, 2012). Literature suggests that cultural diversity is an important criterion to expedite more sustainable food consumption patterns among society (Nicolaou *et al.*, 2009; Schösler *et al.*, 2012). The role of religion in shaping consumers food choice is rather vague except where the impact of food consumption depends on the religion itself

(Bonne *et al.*, 2007). Religion can influence consumers' attitudes and behavior, including food purchasing decisions and eating habits (Pettinger *et al.*, 2004). Eating '*halal*' food by the Muslim community strictly follows the Islamic values as a reflection of obedience and adherence to the religion's beliefs and teachings (Bonne *et al.*, 2007). Muslim consumers' attitudes towards '*halal*' food consumption are influenced by religious belief, mass media and people around them (Aiedah, 2014). Further, the appearance of a religious (*halal*) logo on product packaging helps Muslims to choose and justify their product purchases without hesitation guided by their religious beliefs and laws (Bakar *et al.*, 2013).

Though '*halal*' concept applies specifically to the Muslim society (Alam and Nazura, 2011), there is a huge potential to tap this in to the non-Muslim community as well especially in case of food. The fact that food is a common need for all people, the market potential is even more promising though people from different cultural backgrounds and religious faith do not have same perceptions and experiences to food. In Muslim community, the increasing awareness and concern over health is the basis for acceptance of '*halal*' food as it covers the whole understanding of consuming clean and hygienic food to promote better health. In general, consumers are more conscious of their health which influences their behavior while selecting their food. They search for food with the benefits to keep them healthy and improve their mental state leading to quality of life. The role of food in cultural practices and religious beliefs might be complex; but, it has a unified understanding among Muslims. For instance, the *halal* logo or label helps to convince Muslim consumers that the food product is suitable for their consumption. On the other hand, the non-Muslim consumers understand that food items carrying the *halal* logo are prepared in the most hygienic way. Furthermore, it has also been proven that non-Muslim consumers

do respond positively to '*halal*' food certification (Hasnahet *et al.*, 2009).

Besides the religious value, the other motives behind the *halalan toyyiban* concept include: 1) preserve life, 2) safeguard future generations, and 3) maintain self-respect and integrity (Muhammad *et al.*, 2007). Today, the concept of *halalan toyyiban* is beyond the religious value. Now a day, the rising concern of food consumers is health which could be an untapped opportunity for the '*halal*' food producers. This is because the concern of health due to food consumption basically shares the same value with the *halalan toyyiban* concept. Being healthy means, being watchful over food on the cleanliness, the source, and the method of handling and preparing it. The most important thing is to ensure and minimize any possible harmful effects to the body from the food. There could be several determinants for the market acceptance of the '*halal*' food. It is believed that consumers accept a product when they have the true intention to use it, or have used the product earlier and want to continue in using it. Generally, consumers respond positively to the products with high quality. In the case of food, quality is defined mainly by its cleanliness and freshness. To achieve this, the food processing methods are the key in ensuring the cleanliness and freshness of the food, which can also affect the nutritional value and quality of the food. The food quality is also critical to determine food safety. Grunert *et al.* (1996) classified the food quality dimensions into: hedonic, health-related, and convenience related. They explained: "Hedonic quality is related to sensory pleasure and is therefore mainly linked to taste, smell, and appearance. Health-related quality is concerned with the ways in which consumption of the product will affect consumers' physical health. Convenience-related quality is related to the time and effort which has to be expended while buying, storing, preparing and consuming the product" (Grunert *et al.*, 1996). These explana-

tions, too, relate to the food quality and safety as well. Furthermore, Rezai *et al.* (2011) stressed that the benefits of 'halal' food could be explained from the context of food safety, which is also demanded by non-Muslims.

Studies on the consumers' attitude towards the use of chemicals in agriculture were explored since 1960's (Bearler and Willits, 1968). It marked the beginning of the era when human beings started to be more concerned and aware about preserving the environment. The findings from earlier studies confirmed that consumers showed positive attitudes towards the products of organic farming where one of the most commonly found reasons for choosing these products was - the products of organic farming were perceived as healthier than the conventional counterparts (Chinnici *et al.*, 2002; Harper and Makatouni, 2002). Consumers do not necessarily buy sustainable products due to environmental concern, giving benefit to the community, and personal beliefs; but, mainly to give priority to health (Vermeir and Verbeke, 2004). Researchers have shown that the consumers of organic food are less likely to pay attention to the price as compared to those who do not purchase organic product (Yiride *et al.*, 2005).

In the past two decades, the increased awareness about the environment has had an effect on consumers' behavior, resulting in to expansion of market of the green product at a remarkable rate (Aini *et al.*, 2003). As a result, there is a huge increase in production and consumption of organic products. It is believed that organic products have lesser negative effect to the environment. The National Organic Standards Board of the U.S. Department of Agriculture (USDA), established a national standard for the term 'organic' in December 2000. According to them, organic food is defined by how it cannot be made rather than how it can be made. The organic food must be produced without the use of sewer-sludge fertilizers, most synthetic fertilizers, pesticides, genetic

engineering, growth hormones, irradiation and antibiotics. Many kinds of agricultural products can be produced organically. These include produce of grains, meat, dairy, egg and processed food products. The term 'organic' does not mean 'natural'. There is no fixed definition as to what constitutes a 'natural' food. Nevertheless, the food industry uses the term 'natural' to indicate that a food has been minimally processed and is preservative-free. A natural food can be called as an organic food, but not all natural foods are organic foods.

This exploratory study aims to understand about meaning of organic farming and *halalan toyyiban* foods and find out the relationships between them based on the common characteristics, if any. The key research questions to be addressed in this study were: i. What is our understanding about the organic farming and *halalan toyyiban* foods? ii. What are the common characteristics to relate them, if any?

2. Literature review

For the better understanding of the concept of *halalan tayyiban*, the discussion here starts with the two Arabic words, 'halal' and 'haram'. The 'halal' means to set free, to let go, to dissolve and to allow, or to exit from something that is not allowed (*haram*) (Ibn Manzur, n. d). Alternatively, 'halal' can be defined as something that is allowed and the follower cannot be punished if it is conducted properly (Jayyib, 1998). In other words, 'halal' means anything which is not prohibited or lawful, especially for food and meat from permitted animal which is ritually slaughtered (Cyril, 1989). The opposite of 'halal' is 'haram' (Ibn Manzur, n. d.). It means prohibited, forbidden, unlawful, restricted and or unpermitted (Mohammad, 1993). 'Haram' can be defined as something that must be avoided by the Muslims, and committing the act of 'haram' is sinful and immoral for them (Ibn Hazm, 1983).

Allah s.w.t. (God) commanded specifically on the intake of '*halal*' food, referring to the term '*al-tayyib*' or '*al-tayyibat*' and urging to eat '*halal*' and good quality food and avoiding filthy food. The word '*al-tayyibat*' came from '*taba*' which means good, tasty, delicious, sweet, pure, clean, and free from any materials which are *makruh* (detested) (Ibn Manzur, n.d; al-Ghazzali, n.d). Some Islamic scholars suggested to integrating *tayyib* and *halal* (al-Qurtubi, al-Suyuti, Ibn 'Ashur and Ibn Kathir). This was supported by Sazelin and Ridzwan (2011).

The *halalan tayyiban* concept covers all the necessary factors (physical and spiritual) of the food for the human being. In this connection, the *halalan tayyiban* can be translated as the foods which are permitted (*halal*) for human intake for providing benefits to the human body and mind as well. The food classified as the *halalan tayyiban* should fulfill two criteria: firstly, the food is '*halal*' (and taken from a *halal* source), and secondly, it is a quality food as it provides benefit to human. If the food misses these two criteria, it cannot be called as the *halalan tayyiban*. Hence, it must be avoided by the followers of Islam.

The *halalan tayyiban* also indicates that the determination of '*halal*' food encompasses both the tangible and intangible aspects of the food: Before consumption, the food must be ensured as '*halal*', in good quality, hygienic and safe. These preconditions are applicable from the initial sourcing and handling to the final stage (preparation, manufacturing, storage, distribution and serving). The idea of *tayyiban* does not limit the food to be '*halal*', good, delicious, tasty and pure only. It goes further with the requirement of beneficial and not causing any harm to the body. Al-Ghazali said that "what is beneficial for the body is also beneficial for the mind and soul". In addition, Sazelin and Ridzwan (2011) stated that the good quality food bounded by Islam, also has a relationship in the

development of good quality human capital. *Halalan tayyiban* food should be viewed from the aspect of its complete supply chain, beginning from the farm and reaching up to the dining table. This means, it is important to ensure that during the whole process, the food should not be contaminated by anything which may be harmful to the human health.

As underlined by the Syariah law, the term '*halalan tayyiban*' refers to the products which are safe to be consumed (Omar *et al.*, 2013). As Allah s.w.t. (God) says in the Quran, 'O mankind! Eat of that which is lawful and good on the earth' (Surah Al Baqarah 2: 172). They ask you (O Muhammad SAW) what is lawful for them (as food) ... Lawful unto you are at Tayyibaat (all kind of '*halal*' foods) (Surah Al Maidah 5: 4). As explained, Islam requires that Muslims find *rizk* (sustenance) and consume food that is *halalan tayyiban* because it ensures a healthy living that reflects good attitudes and behaviors as well (Yousef, 2010). It goes further by covering the concept of wholesomeness, which includes quality, cleanliness, and safety of the food (Omar *et al.*, 2013).

The results from an earlier study suggest that non-Muslim consumers are aware of the existence of '*halal*' food in Malaysia. In general, socio-environmental factors such as socially mixing (of non-Muslims) with Muslims and the presence of advertised '*halal*' food significantly influence non-Muslims' understanding of the '*halal*' principle. These findings also suggest that non-Muslims understand that '*halal*' principle that addresses the issues of food safety and environmental friendliness. In the study, at least 94 percent non-Muslims agree that the '*halal*' principle is religious obligation, while 90 percent and 71 percent agree that it is concerned with food safety and environmental friendliness, respectively (Rezai *et al.*, 2012). Therefore, these suggest that a close relationship exists among *halalan tayyiban* food safety and

environmental friendliness, which are also the essential characteristics of the products from organic farming. In an earlier and related study, we have highlighted that sustainable agriculture can be achieved through organic farming (Othman and Hasan, 2016).

3. Methodology

This study employed a qualitative research using the face-to-face interviews and secondary data approaches. The interviews were conducted with six respondents who also allowed the interviews to be recorded. Later, phone calls were made to the selected respondents to obtain clarity of the information from them. The location of this field study was at Sri Lovely Farm, Sik, Kedah, Malaysia. The interviews were conducted with the managing director (Farmer 1), his two assistants (Farmer 2 and Farmer 3), a volunteer (Farmer 4), and two interns by visiting the farm on October 24, 2016.

Traditional and computer-based qualitative methodologies were used to analyze the data and to compare and contrast the observation. The method suggested by Corbin and Strauss (1990) was used in the data analysis. All data were first reviewed and then categorized.

4. Results

4.1. Characteristics of halalan toyyiban

The characteristics of *halalan toyyiban* can be divided into four: 1) Quality, 2) Healthy, 3) Clean, and 4) Environmental friendly, which are further explained below.

4.1.1. Quality

The whole process of '*halal*' accreditation is stringent; therefore, it has some beneficial characteristics which can also be enjoyed by non-Muslim consumers. Its requirements meet many of the conventional quality standards, like ISO, Codex Alimentarius, Hazard Analysis and Critical Control Point, and Good Hygien-

ic Practice. Therefore, implementing the *halalan toyyiban* requirements (to obtain '*halal*' accreditation) should ensure to produce higher quality food products (Talib and Ali, 2009). Considering this, the '*halal*' values may become popular among non-Muslim consumers, if the society at large is made to be more aware of issues concerning health, hygiene, safety, environment, and animal welfare which come along with the '*halal*' ways of doing the things.

4.1.2. Healthy

Halalan toyyiban foods are those, which have been handled and prepared by following the strict hygiene, and the high standards of nutrition, cleanliness and safety. In other words, the food must be produced and handled by fulfilling the stringent requirements of the Islamic Dietary Law, which as a result guarantees that the food is healthy. Since more and more people are becoming health-conscious, the *halalan toyyiban* principles of preparing food may no longer remain confined to the strictly religious need but may become an alternative to non-Muslims for a healthy life.

4.1.3. Clean

Allah's s.w.t. (God) command to select *halalan toyyiban* food can be seen in the verses of the al-Quran, and among several one is surah al-A'raf (7) verse 157. In this, the word '*al-tayyibat*' is interpreted as '*halal*' (al-Qurtubi, n. d.; al-Tabari, n. d.; al-Suyuti, 1990); '*halal*' and not repugnant (Ibn 'Ashur, 1984). One more interpretation is: '*halal*' is good, beneficial to the body and helpful in terms of habits and the law of Islam (Ibn Kathir, n. d.). Also, "*tayyib*" is mentioned in surah al-Baqarah (2) verse 168. Furthermore, al-Sharbini (n.d) explained that the "*toyyiban*" has four principal elements as listed below:

- i) Both the source and whole content of food is '*halal*', no haram is included
- ii) It is clean, therefore, does not contain any impurities

Table 1: Key differences between conventional and organic farming-Sri Lovely Farm focusing on the seed used

Key practices	Conventional	Sri Lovely Farm
1. Seed preparation	Seed not selected	Seed selected: Seeds soaked for 24 hours prior to sowing to eliminate non-viable ones
2. Quality of seedling at transplant	All kinds of seedlings	Only healthy seedlings transplanted

iii) It does not cause any negative effect upon intake

iv) The food contents are nutritious; therefore, beneficial to human.

4.1.4. Environmental friendly

Taking the paradigm shift into account which emphasizes on the need of the green supply chain, the 'halal' principles are no more only for the Muslims of slaughtering permitted animals in the Islamic way. It also emphasizes on the sustainability, environmental friendliness, food safety and animal welfare. Hence, the 'halal' standard implies the Halalness of the products to Muslims and it stands for not only just and fair business transactions but also caring for the environment, sustainability, and animal welfare.

4.2. Characteristics of organic farming

The characteristics of organic farming can be divided in to four: 1) Healthy seed, 2) Natural fertilizer, 3) Quality soil, and 4) Natural insect control. These characteristics are further explained below.

4.2.1. Healthy seed

The seeds being used in Sri Lovely Farm are of a very good quality. As depicted in the Table 1, only viable seeds are selected after soaking in water for 24 hours followed by sowing in the container for 4 days before planting on the ground. The seeds are free of GM seeds. The concept of the healthy seed selection can be related with the quality of produce (in this case rice). "Original seed instead of the modified seeds". The original seeds are

hereditary which, when we planted the seeds can later use to return to replanting"- (Farmer 4, personal communication, October 24, 2016)

4.2.2. Natural fertilizer

Fertilizers used in Sri Lovely Farm are natural, from 100% natural ingredients produced locally, and which are organic entirely. Fertilizers produced in Sri Lovely Farm are from rice straw, and fruit waste collected.

4.2.3. Quality soil

The quality of the soil in Sri Lovely Farm is monitored by the Department of Agriculture, Malaysia in order to maintain soil quality of the farm land.

"Our soil was often taken to be used as a sample, enter the lab. From there, we could know the soil contains heavy metal or not" - (Farmer 1, personal communication, October 24, 2016)

The soil/land of high quality can produce rice of high quality, and also help in balancing the ecosystem, which is good for the environment. Quality of soil is maintained by the nutrients - carbon (C), hydrogen (H), oxygen (O), Potassium (K), calcium (Ca), magnesium (Mg), sulfur (S), Phosphorus (P) and nitrogen (N). Carbon, hydrogen, oxygen and nitrogen can be obtained from the air whereas; potassium, calcium, magnesium and sulfur are usually obtained through fertilizers. Fertility of the soil is very important and it is one of determining factors of the crop yield.

4.2.4. Natural insect control

Insect control is being practiced by Sri Lovely Farm in an ecofriendly way to avoid the synthetic and dangerous chemicals.

“We have own methods, so how we want to control of caterpillars, we use 'ubi gadung', so, we use the traditional concept of back to nature” - (Farmer 1, personal communication, October 24, 2016)

According to the one of respondents from the Sri Lovely Farm, the insect control is being carried out by using 'ubi gadung' (*Dioscorea daemona*).

5. Discussion

This study was exploratory with an aim to further understand about the food choices classified under “organic” and “*halalan toyyiban*”. To our surprise, it was found that the key issues of environmental friendliness and food safety were addressed by the ‘*halal*’ principles as revealed by the non-Muslims. Considering about our future generations, we must put in our best effort in promoting and maintaining a sustainable green environment, these issues are very critical in that sense. And the *halalan toyyiban* food helps by providing a choice to consumers to meeting the sustainability goal. We al-

ready know that some non-Muslim consumers are familiar with the ‘*halal*’ principles and food products available in the market. To make it further successful, more awareness promotion about the *halalan toyyiban* food is needed by emphasizing that it’s not only about the religious point of view but also about the common benefits for all such as the food safety, wholesomeness, hygiene, caring for animal and environment. In this effort, the Muslim consumers have a key role to play by promoting and making their non-Muslim friends aware about the *halalan toyyiban* principles of producing the food.

To the best of our knowledge, this study was the first attempt to relate the foods of *halalan toyyiban* with organic farming. Therefore, enough information was not available in the published form especially when it was about to establish the relationship between the *halalan toyyiban* and organic farming. However, it was possible to collect some materials relevant which were found separately under *halalan toyyiban* and organic farming. By combining our insights obtained from both primary and secondary data, we have been able to come up with three common characteristics to show the significant inter-relationships between *halalan toyyiban* and organic (farming) foods. These are: 1) quality; 2) healthy; and 3) environmental friendly (natural). This is further illustrated in the Figure 1.

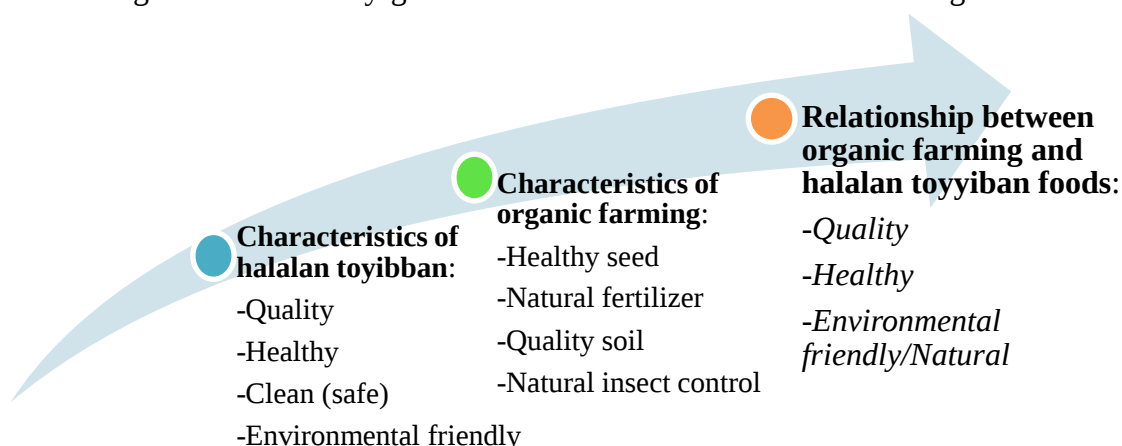


Figure 1: A proposed model to show relationship between organic farming and *halalan toyyiban* foods

6. Conclusion

We conclude that there is a significant relationship between the integral part of the *halalan toyyiban* principles and practices of organic farming to produce food. At least three characteristics namely, 'quality', 'healthy' and 'environmental friendly (natural)' were identified in common between *halalan toyyiban* principles and practices of organic farming. These insights might help in the value proposition of the both kind of produce and products to all consumers regardless of their faith and religion. Furthermore, Malaysia, as the pioneer and promoter of the *halalan toyyiban* food, should be able to further promote and successfully enter in to the emerging global market with its own produce and products by utilizing insights reported in this article.

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Biotechnological Approaches: Sustaining Sugarcane Productivity and Yield

Ashutosh Kumar Mall and Varucha Misra*

ICAR-Indian Institute of Sugarcane Research, Lucknow- 226 002, Uttar Pradesh, India;

*Correspondence: Ashutosh.Mall@icar.gov.in / misra.varucha@gmail.com; Tel: +91 522-2480726

Abstract: Biotechnology is an important field of science which is playing a vital role in agriculture and other domains. The idea of creating new hybrid varieties is not new; however, earlier this process was possible only in close species associated with each other. With the use of biotechnological techniques, it is now possible even in species which are not closely associated. Sugarcane crop is also not left untouched by this field of science. It has paved a new way for improving the cane production and productivity. It even helps in enhancing the sucrose content of the crop. Sugarcane researchers have achieved success in several aspects with the use of these techniques like developing high yielding cane varieties; enhance accumulation of sucrose content in cane stalks, *etc.* Although there are still certain constraints which have yet not been solved in this crop but the way field of biotechnology is developing, it is not far that these constraints will also be overcome. In this article we are highlighting the usefulness and potential of biotechnology approaches to boost the sugarcane productivity and yield for the sustainability.

Keywords: Abiotic stress; high yielding; productivity; sugarcane

1. Introduction

Sugarcane is a crop that imparts sweetness to human's life. It is a major sugar producing crop that contributes to more than 70 per cent for production of sugar. It covers an area of around 3.8 million hectares with an annual cane production of around 270 mt. 2.8 per cent of the cultivated land area is occupied by this crop and in respect to agricultural production about 7.5 per cent is contributed by this crop to India. In India, 42.02 (%) and 57.98 (%) is contributed to sugarcane area in tropical and sub-tropical zone, respectively, while in terms of production it is 48.58 (%) and 51.42 (%), respectively (Shukla *et al.*, 2016). It is well known that this crop is a major source of food as well as fuel production. The new field of biotechnology has the power of improving cane production as well as yield. Using

this new field of science as a tool, researches specialized in plant breeding can be able to produce better crops. The technologies used in this field have the capability to transfer and alleviate a single gene/or number of genes of desired trait rather than thousand of genes from one species to the other one (Nel, 2009).

Biotechnological approaches in the plant kingdom have been playing a significant role from past many decades. This field of science has encompassed the magnificent genetic engineering developments within itself in several folds. The crops obtained from such methods have been known to be the latest technological approaches that have helped in boosting up the production of food to a great extent. These transgenic crops have many beneficiary points like easier application of herbicide and that too in very low levels as per the normal practices which in turn

helps in reducing the cost of production as well as in overcoming the environmental pollution (Baker and Pretson, 2003). In case of sugarcane crop, on worldwide basis there is high pressure to augment cane productivity for sustaining the profits of sugar mills (Halon *et al.*, 2000). In this regards, various sugarcane researchers have been showing effort in developing new hybrid cane varieties that possess high yield and high sugar contents under conventional breeding programmes of sugarcane at different institutes. With the use of these approaches, new cultivars are being able to develop which possess high sugar content, better ability of ratooning as well as resistance towards various diseases. These newer techniques and methodology have paved new way in the field of breeding for improving varieties and also helped in rapid multiplication of these varieties. A common breeding constrain in developing new varieties is its slow multiplication rate as well as its rapid spread. This creates a problem in not fulfilling the seed requirement of the newly developed varieties, biotechnology in this aspect, had helped in faster multiplication of new varieties (Source access: http://shodhganga.inflibnet.ac.in/bitstream/10603/42274/7/07_chapter%202.pdf, 3.05.2017).

2. Biotechnological achievements

2.1. In improving cane production

Being a major food and fuel source all over the world, biotechnology in this regard has the power for improving the economically important traits in this crop. The key approach in improving sugarcane production lies in the classical plant breeding method but plant breeders always encounter difficulty in this regard as cane genome is highly complex and possess narrow genetic base (Roach, 1989; Lima *et al.*, 2002). The biotechnological approaches had successfully improved cane production especially the inter-specific *Saccharum officinarum* and *S. spontaneus* hybrids (Usman, 2015).

Another most important constrain is the time required for a new variety to develop and commercialize that generally takes a long time of 12-15 years. As mentioned before that biotechnology helps in transferring a desired trait of gene from one plant to other so in case of sugarcane crop, there is certain desired traits which would not be able to introduced into it through the normal plant breeding methods. The victory of improving the crop production by biotechnological tools lies in the high levels of the trans-gene expression. In this aspect, promoters have been identified in driving the high levels of gene expression in transgenic sugarcane, particularly in stem and leaves. The first identified promoter was obtained from Cestrum yellow leaf curling virus which impels the elevated level of constitutive trans-gene expression significantly higher than the ones obtained by the maize *polyubiquitin-1* (*Zm-Ubi1*) promoter (a well known benchmark). Another identified promoter was the maize *phosphoenolpyruvate carboxylate* promoter which facilitates the expression levels, particularly in the leaf region of sugarcane, compared to *Zm-Ubi1*. By the process of gene modification, the transgenic expression was enhanced by approximately 50-fold for better cane production (Kinkema *et al.*, 2014). Bower and Birch (1992) had achieved success in the sugarcane transformation trailing with the development of micro-projectile system. Some studies had showed improved resistance in developing a transgenic sugarcane crop towards micro-organisms acting as pathogens (Joyce *et al.*, 1998a, b; Ingelbrecht *et al.*, 1999; Zhang *et al.*, 1999; Gilbert *et al.* 2005;), towards pests like stem borer (Arencibia *et al.*, 1999; Braga *et al.*, 2003) and towards herbicide (Gallo-Meagher and Irvine, 1996; Enriquez-Obregon *et al.*, 1998).

The use of techniques of genetic engineering in the past two decades by plant breeders had caused transmission of noble gene into the plant for developing

better characteristics in them. The technique involves insertion of foreign genes into the parent plant, use of protoplasmic cells or tissues for the development of transgenic plant having normal physiological and biological functions. Several advances have been seen from past several years in field of molecular biology as well as genetic engineering pertaining to crops (Jenes *et al.*, 1993). With the use of a technology based on recombinant DNA it is now even achievable to clone a gene, modify or mobilize it and even integrate it in any other without any discrimination from where the gene has been taken (Chakrabarty *et al.*, 2002). About 50 % losses are being occurring by different types of borers but the use of *Bacillus thuringiensis* by these technologies have shown harmful effects towards these borers. In the present scenario, by the use of cry genes, greater than 30 species of different plants have been transformed (Schuler *et al.*, 1998).

2.2. In developing high yielding sucrose cane varieties

Biotechnological approaches had made possible in increasing the frequency of capability of parental clones possess with very high sucrose content which in prevailing breeding programmes is at a very low level. In case of sugarcane crop, Sugarcane Breeding Institute had initiated programme in this aspect. The target of this programme is to develop genetic stocks that consist of high sucrose by the use of recurring cycles of intensive crossing and selection. In cycle I, about 5420 seedlings obtained from 30 bi-parental crosses were considered. Out of all the crosses performed, crosses between CoC 671 x CoT 8201, Co 86002 x Co 62198, Co 85002 x CoT 8201, CoC 671 x Co 94019, PR 1080 x Co 94008 and PR 1080 x CoT 8201 had shown high levels of sucrose contents. The approach of biotechnology in this aspect of developing high sugarcane varieties have opened up a new option which will reward to sugar millers in two ways, firstly productivity will

raise and secondly cost of production will be reduced (Shanthi, 2016).

2.3. Towards abiotic stress

For tolerance to a particular stress or multiple stresses, biotechnology had showed a new way for developing transgenic plants. The best short-term approach for development of stress tolerant cane variety lies on the base of selection and breeding wherein wide crosses are being made. For the development of the stress tolerant variety certain steps have been outlined (Figure 1) (Epstein and Rains, 1987). Researchers had identified candidate genes in sugarcane for imparting tolerance to various abiotic and biotic stresses. In this view, some of the examples of candidate genes which have been identified in case of drought/water deficit condition are DREB (dehydration responsive transcription factor), HSP (heat shock proteins), LEA (late embryogenesis), RAB (responsive to abscisic acid), osmotin, choline oxidase and annexin (Nair, 2011), stress-related clusters showing differential expression (>2-fold) during biotic and abiotic stress conditions (Gupta *et al.*, 2010), sugarcane ethylene-responsive factor, SodERF3 (Trujillo *et al.*, 2009), up-regulation of genes regulating intracellular redox status (Prabu *et al.*, 2011) and presence of LEA (late embryogenesis abundance)-related proteins and dehydrin (Iskandar, *et al.*, 2011), accumulation of trehalose and proline (Molinari *et al.*, 2007; Guimarães *et al.*, 2008), other stress-inducible proteins (Jangpromma *et al.*, 2010), early response to dehydration protein 4 (ERD4) (McQualter *et al.*, 2007). Wahid and Close (2007) had identified various expressions of genes or proteins in sugarcane grown under temperature and salinity induced stress. Some of the instances of such gene expression under stress are heat stress-induced DHNs (Wahid and Close, 2007), genes encoding for $O^{\cdot-}/OH^{\cdot}$ radicals and reduction of H_2O_2 by peroxidase/ catalase under heat stress (McQualter *et al.*, 2007; Chagas *et al.*,

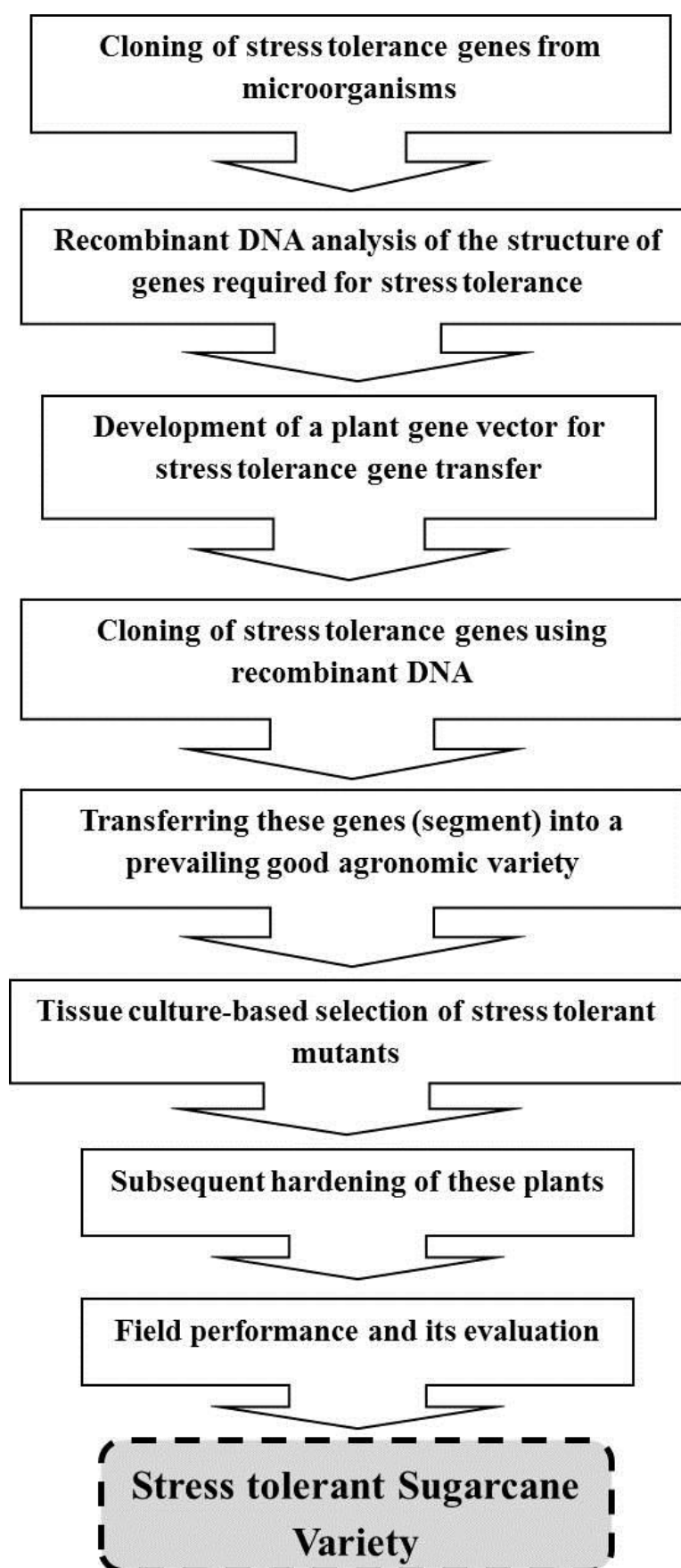


Figure 1: A diagrammatic sketch depicting the steps involved in using molecular approaches for the development of stress tolerant sugarcane variety (Source: Data from Shrivastava *et al.*, 2016).

2008; Shrivastava *et al.*, 2012), cold-inducible ESTs, PPDK and NADP-ME proteins and dehydrin-like proteins which protect membranes against chilling stress (Nogueira *et al.*, 2003), reduced activity of sucrose phosphate synthase, NADP-MDH and pyruvate orthophosphate dikinase to maintain photosynthesis under chilling damage (Du *et al.*, 1999), induction of Galactinol synthase (GolS) and pyrroline-5-carboxylase synthetase (P5CS) (McQualter *et al.*, 2007) and osmolytes like proline and glycine betaine (Patade *et al.*, 2008) during salinity-induced stress. Shaik *et al.* (2007) had mediated transformation in sugarcane through a microorganism *Agrobacterium tumefaciens* with two plasmid LBA4404 pB1 121 construct GLY1 that bestowed stress tolerance in crop. Another bacterial transformation of *A. tumefaciens* imparted tolerance to drought and salinity in sugarcane using Arabidopsis Vascular Pyrophosphatase (AVP1) gene (Kumar *et al.*, 2014).

Some recent achievements in providing tolerance to abiotic stress in sugarcane through biotechnological tools are as follows:

- i. Identification of nearly 600 differentially expressed genes in cane grown under low temperature for activity of the trans-membrane transporter with an enhancement of ~2.5 fold in *Ssp-NIP2* expression (*Saccharum* homolog of a NOD26-like major intrinsic protein gene (Park *et al.*, 2015).
- ii. For enhancing tolerance power of cane towards drought and salinity, over expression of PDH45 (a DEAD-box helicase gene- a pea isolated gene) in transgenic sugarcane. This gene also exhibited an up-regulation of DREB2-induced downstream stress-related genes (Augustine *et al.*, 2015). Another expression of genes in response to drought in sugarcane, expression of a set of genes majorly accountable for synthesis or expression of trehalose 5-PO₄ and sucrose-PO₄ in

response to foliar application of salicylic acid (Almeida *et al.*, 2013).

- iii. Identification and expression of genes related to defense/ signaling sequences in smut and eyespot disease inoculated cane plants- 62 differentially expressed genes having 19 transcript derived fragments (TDFs) and a chitinase gene *ScChi* which is concerned in interaction of host with pathogen (Borrás-Hidalgo *et al.*, 2005; Que *et al.*, 2014).
- iv. Identification and expression of EST clusters that are responsible in signaling of reactive oxygen species (ROS), defense response and sugarcane innate immunity against red rot infection (Sathyabhama *et al.*, 2016).
- v. Development of drought tolerant transgenic sugarcane- PT Perkebunan Nusantara in Indonesia, University of Jember (East Java) and Ajinomoto Co., Inc., Japan had developed this transgenic plant by using *bet A* gene from the *Rhizobium meliloti* that produces glycine-betaine. This product is an osmo-protectant which imparts tolerance towards drought stress. This GM transgenic cane produced 20-30 per cent more sugar in comparison to other cane varieties opted for drought conditions (Marshall, 2014; Waltz, 2014). With the approval by the national genetically Modified Products Bio-safety Commission of Indonesia this has gained the first position of the world's first commercialized GM sugarcane (Anon, 2013).

3. Sugarcane production and productivity

Biotechnology has paved a new way for improving the cane production and productivity. To increase the sucrose content in the crop, genetic manipulation are being used which requires a complete knowledge and command in the processes involved in sucrose accumulation within the cane stalks (the storage house of the plant). Researchers have been successful

in identifying the enzymes that gives a start to these processes, however, through the modern technique of genetic engineering these enzymes can be hasten or slowed down to attain more efficient storage and accumulation of sucrose in cane stalks. The application of genetic manipulation in cane stalks are being conducted one step at a time. Towards first step for success South African scientists had knocked down a certain enzyme by genetic means which enhanced the sucrose content in young stalks of sugarcane (Groenewald and Botha, 2008). Another perspective is making cellulosic bio-fuel production easier. It is well known that the sucrose is an essential component for production of bio-ethanol, an alternative for fossil fuels, through the process of fermentation. Breeders are focusing now on enhancing the sucrose yield for increasing production of ethanol without compromising the sucrose content as food commodity. By the modern use of biotechnology tools the cellulose content in cane leaves as well as bagasse are being used for the production of ethanol thereby not utilizing the main cane product, sugar. The intricate structure of cellulose can be broken down into simpler molecules of carbohydrates by number of enzymes which later can be used for production of ethanol by fermentation nevertheless cellulosic structure is guarded by lignin. This hard guarding material requires a costly procedure for its removal. Brazilian scientists are taking initiatives through genetic engineering in modifying the cellulosic structure so that it could be separated from bagasse without any difficulty (<http://agencia.fapesp.br/en/16756>, accessed on 03.04.2017). Adding to it, Australian researchers developed transgenic canes by inserting genes capable of producing the cellulose degrading enzymes in leaves of mature plants (Harrison *et al.*, 2011). Besides production of small products through sugarcane bio-factory which involves the tweaking of genetic mechanism occurring in cells of sugarcane plant that instructs them for the production of

these small products which in turn converts the complete cane plant into a bio-factory. A number of high value products are being produced like therapeutic proteins (Wang *et al.*, 2005) and biopolymers (Petrasovits *et al.*, 2007; McQualter *et al.*, 2005). Another important production of small product is production of isomaltose which was possible by insertion of a bacterial gene into the cane plant for production of an enzyme responsible for conversion of sucrose into iso-maltose, an alternative sweetener (Wu and Birch, 2007). Biotechnological efforts gave positive results in stream of characterization of genome structure, specific traits mapping, marker assisted selection in resistance towards insect/disease, variability of pathogens on molecular basis, transformation, pathogen detection in precise manner in plants and many more. The sharpness in sensitivity of the assays made them more rapid for routine analysis of plant pathogen detection and identification. Besides, the assay has become even more economical. The better ability to detect the infection at early or latent stages help in improving the management of disease as well as restricting the movement of the various diseases and even assist in solving the phylogenetic relationship amongst the various pathogens. This also assists in developing newer strategies for enhancing the resistance activity of the host by genetic transformation methods. There is a need to improve the technologies for development of transgenic before making it a part of the varietal developmental programmes of sugarcane. There is still a challenge for the researchers in developing a pathogenic free transgenic as this requires complete understanding of interaction of plant and pathogen, however, gene cloning, to some extent, is making a new revolution in this aspect. There is still a need to identify and characterize the genes responsible for the antifungal activity in case of disease resistance. In grassy shoot disease of sugarcane and Sugarcane yellow leaf syndrome, a common occurring disease

development of PCR diagnostic kits are needed for Phytoplasma.

For improving the crop quality as well as productivity and even providing resistivity to pathogen, tissue culture is playing an effective role. The use of cryotherapy has enhanced the likelihood of attaining healthy plants. In case of attaining virus resistant plants, micropropagation is the best tool which requires the use of shoot apical meristem (acting as explants). In minimizing the somaclonal variation in times to come, application of clonal propagation as well as research in transgenic are needed. There is a need to work on the structure of genomes so that identification of markers associated with important agronomic characters may be performed. Transgene constructs that help in lessening the hazardous effect of environment and even bio-safety jeopardize of these plants are need of the upcoming times. The way researchers are moving towards the biotechnological techniques many mysteries will unravel (Tiwari *et al.*, 2010).

4. Biotechnological challenges in enhancing the cane production and productivity

The use of biotechnology in sugarcane crop has drawn researchers as well as entrepreneurs towards itself but its application over this crop on commercial basis had always be a regulatory challenge particularly in case of field cultivation. There is higher probability of transferring of genes along with unwanted genes from the source plant to other plants used as a food commodity. Therefore, the practicability of these techniques on commercial cane bio-factory will be dependent on how much amount of risk containment as compared to the non-food product plants, for example tobacco (<http://isaaa.org/resources/publications/pocketk/45/default.asp>, 27.3.2017). A commonly used technique now-a-days is tissue culture technique that helps in developing uniform disease free plantlets in

short time, however, a major problem in this technique is its cost of production (Usman, 2015).

One of the most talked topics in biotechnology achievements is the development of transgenic varieties and the area under the transgenic obtained plants has increased to approximately greater than 81 million hectares but there are certain limitations too in developing it. Singh *et al.* (2013) showed that the drawbacks in developing cultivars are not even removed by the process of transformation; however, other methods for precise integration and control trans-gene expression are still to be performed in sugarcane crop. Furthermore, in studies related to sugarcane association, there is still need for developing high throughput markers as well as producing more markers and even ensure the proper availability of these markers. The newly developed markers will enhance the knowledge and mystery of complex structure of sugarcane genome. DNA-based molecular markers of progenitor plants have the potential to show the prevailing genetic polymorphism that may be helpful in case of this crop as parental genome is much less complex in comparison to the hybrids ones (Henry *et al.*, 2012).

5. Constrains in improving cane production using biotechnological tools

Biotechnology is generating enormous information which had played an important role in transforming the world of science. Biotechnological interference in sugarcane crop provides a chance for sugar producers and cane farmers to enhance the production and sustainability. These approaches act as a proactive approach in alleviation of the troubles occurring in cane production (Usman, 2015). Though researchers are attaining much success in improving the cane production and yield yet there are certain constrain in this regard using biotechnological tools. Some of these constrains are: High-throughput sugarcane

transformation that involves efficiency of the low transformation, inactivation of trans-gene, soma-clonal variation, and the long time required for regeneration and its commercial release. In restricting the access in exploiting gene technology, transformation and tissue culture-induced soma-clonal variation are the two things that remain important for sugarcane improvement (Arencibia *et al.*, 1997). The substantial alteration in the current transformation systems are required to make sure of the clonal fidelity of transgenic cultivars. Also as stated before the time lag of developing and releasing a cane variety is also a crucial challenge as well as constrain for cane breeders. At times the cane grown and developed by classical method yields similar/or enhancing gains as compared to the ones developed through biotechnological approaches. There are two major factors, viz., polyploidy and aneuploidy that cause molecular characterization of cane genome more difficult. Aitken *et al.* (2014) had reported that the recent available genetic maps and markers obtained from sugarcane provide partial information in aspect of genome organization. The reason behind is the low density of markers and its coverage. This causes difficulty in allocating the markers into linkage groups (Souza *et al.*, 2011).

6. Concluding remarks

This chapter concludes that various successful achievement have been attained by plant breeders by using the biotechnological techniques in this crop but the way, application of biotechnology is spreading its hands in agriculture especially in sugarcane crop, the left over mysteries could be unravelled much easier in the times to come. Progress in traditional breeding of sugarcane, a highly polyploid and frequently aneuploid plant, is impeded by its narrow gene pool, complex genome, poor fertility, and the long breeding/selection cycle. These constraints, however, make sugarcane a good candidate for molecular breeding.

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Bioremediation: A Biotechnology Tool for Sustainability

Niharika Chandra, Ankita Srivastava, Swati Srivastava, Shailesh Kumar Mishra and Sunil Kumar*

Faculty of Biotechnology, Institute of Biosciences and Technology, Shri Ramswaroop Memorial University, Barabanki, Uttar Pradesh, India; *Correspondence: sunil.bio@srmu.ac.in / sunilsbt@gmail.com

Abstract: The term bioremediation refers to the use of natural biological agents such as microbes (bacteria, fungi, and yeast), plants or the enzymes released by them to return the polluted natural environment to its original uncontaminated state. Bioremediation is a novel, safe, cost effective, and ecologically feasible technology to detoxify accumulated pesticides, toxic chemicals, fertilizers, aromatic compounds, xenobiotics, hydrocarbons (oil spills), heavy metals in soil and water. Both *in situ* and *ex situ* bioremediation are being used at a large number of sites all around the world with varying level of success. Although bioremediation cannot degrade all the toxicity, particularly all the inorganic contaminants, but it is still more eco-friendly and less expensive as compared to other remediation methods like incineration, chemical treatment and thermal recovery of pollutants. Furthermore, advances in bioremediation are being achieved by coupling this biological method with molecular, genetic engineering, microbiology, pathway engineering, and enzyme design and immobilization tools. In this chapter we have discuss the general process for bioremediation, *in situ* and *ex situ* classes of bioremediation followed by the types of bioremediation techniques being used till date. The role of bioremediation to deal with different types of pollutions and comprehend the advantages, disadvantages and sustainable use of its approaches is also highlighted.

Keywords: *Ex situ* bioremediation; GMOs; *in situ* bioremediation; microbes; pollutants

1. Introduction

A rapid increase in human population accompanied with technological advancements in fields related to agriculture, industries and health has led to accumulation of various toxic chemicals and xenobiotic compounds in our environment. Indiscriminate use of fertilizers and pesticides in agriculture, poor handling of wastewater and solid waste, untreated release of polluted discharge from industries has led to shortage of clean water sources and disturbances in soil content and quality (Kamaludeen *et al.* 2003). Contamination of soil with heavy metals, xenobiotic compounds and municipal waste is responsible for loss of

biodiversity and functional aspects such as cycling of nutrients (Su *et al.*, 2014). Similarly, increased pollution in aquatic ecosystem is resulting in decreased purity and content of ground water as well as surface fresh water (Zhang *et al.*, 2011). Hence, we are in urgent need to seek a feasible and efficient system to manage such pollutants.

Bioremediation is the use of living organisms, primarily microorganisms, to degrade the environmental contaminants into less toxic forms. The process involves the degradation or detoxification of hazardous substances, which are harmful to human health and/or the environment, with the help of naturally occurring bacteria, fungi and plants. Microorganism which perform the

function of bioremediation are known as Bioremediators (Kumar *et al.*, 2011). Bioremediation is a natural, effective and environment friendly alternative to previously used methods for degradation of harmful contaminants, such as physical removal, absorbents, catalytic destruction, incineration etc. which are high cost and nonspecific methods (Head and Swannell, 1999; Gillespie and Philp, 2013)

Although use of microbial consortia have proved their capability for remediation, application of biotechnology and genetic engineering tools is further improving the efficiency and decreasing the cost involved in treating toxic substances. Bacteria, fungi, yeast and algae along with several plants are being used for this purpose.

We will discuss the general process for bioremediation, *in situ* and *ex situ* classes of bioremediation followed by the types of bioremediation techniques being used till date. The role of microorganisms in bioremediation as well as their genetic modification by the use of recombinant DNA technology will be understood. Further we will discuss the role of bioremediation to deal with different types of pollutions and comprehend the advantages, disadvantages and sustainable use of bioremediation approaches.

2. Bioremediation

Bioremediation is the branch of biotechnology that deals with the solutions of problems related to the environment. Bioremediation also involves the process of cleaning the environment from different types of pollutants as well as contaminants by using bacteria, fungi etc. Bacteria play a vital role in the process of bioremediation since it break down the dead materials into organic matter and nutrients. Several types of contaminants such as chlorinated pesticides etc. can be easily digested by

bacteria. Also oil spills can be treated by bacteria (Agarwal and Liu, 2015).

Recently it has been noticed that the awareness of the dangers of many chemicals used in our society has led to research on formulation of products that are more easily degraded in the environment. The process of bioremediation involves the degradation of contaminants using microorganisms that have adverse impact on environment and humans. Bioremediation includes the actions of several microorganisms that are acting in parallel or sequence, in order to complete the process of degradation. In this process, both *in situ* as well as *ex situ* remediation are used. Hence, bioremediation is a technology applied in case of different environmental conditions where the numerous and versatile microbes degrades a vast array of pollutants (Majone *et al.*, 2015).

On the basis of ecological point of view, the term bioremediation involves the interactions between three factors that is substrate (pollutant), organisms, and environment, as shown in Figure 1. The interactions between these three factors primarily affect the biodegradability and bioavailability of pollutants, and physiological requirements of microbes, which plays a vital role in the assessment of the feasibility of bioremediation. Biodegradability defines whether any chemical can be degraded by microbes or not, whereas bioavailability refers to the availability of a pollutant to organisms that are capable of degrading it. For instance, the substrate has low bioavailability if it is tightly bound to soil organic matter or it is trapped inside its aggregates. The conditions that are required by microorganisms to carry out the process of bioremediation include different factors like nutrient availability, optimal pH, and availability of electron acceptors, such as oxygen and nitrate etc. are referred to as physiological requirements.

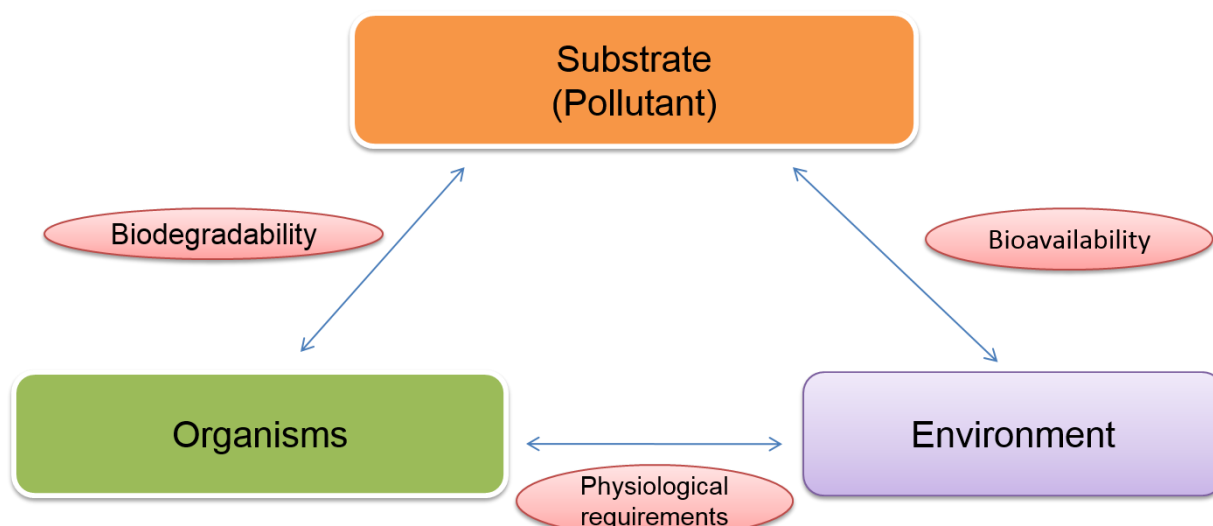


Figure 1: Bioremediation from an environment perspective.

Table 1: Summary of bioremediation approaches

S.N.	Approaches	About	Advantages	Disadvantages	Examples
1.	<i>In situ</i>	At the site	Non-invasive Most cost efficient Relatively passive Treats soil and water Can be done on site	Monitoring difficulties Extended treatment time Environmental constraints	Bioventing Bioaugmentation Biosparging
2.	<i>Ex situ</i>	Away from the site	Low cost Time efficient	Space requirements Need to control abiotic loss Bioavailability limitation Mass transfer problem	Landfarming Biopiles Composting
3.	Bioreactors		Rapid degradation kinetic Enhances mass transfer	High cost capital High operating cost	Slurry reactors Aqueous reactors

3. Classes of bioremediation

3.1. *In situ* bioremediation (ISB)

In situ bioremediation (ISB) is the class of bioremediation that is performed at the original site of contamination. There is no excavation or removal of polluted soil/ground water to any secondary location for conduction of remediation process. *In situ* remediation includes techniques such as bioventing,

biosparging, bioslurping and phytoremediation along with physical, chemical, and thermal processes. This class of bioremediation technology is beneficial because of its low cost, more effective method as an alternative to the standard pump and treats methods that are used to clean up aquifers and soils contaminated with organic chemicals including fuel hydrocarbons, chlorinated solvents. ISB has the potential to provide advantages such as complete

destruction of the contaminants, lower risk to site workers, and lower equipment or operating costs (Koning *et al.*, 2000; Vidali, 2001).

3.2. *Ex-situ* bioremediation (EXB)

Ex-situ bioremediation (EXB) is defined as a biological process which involves excavation of polluted soil or pumping of groundwater that is placed in a lined above-ground treatment area to facilitate microbial removal of contaminants. *Ex situ* remediation includes techniques such as Landfarming, biopiling, and processing by bioreactors along with thermal, chemical, and physical processes (Koning *et al.*, 2000). *Ex situ* remediation is a more thorough remediation technique, but due to the costs associated not only with the remediation processes, but also with the excavation and transportation of the soil, many people are looking towards *in situ* remediation techniques (Vidali, 2001) as depicted in Table 1.

4. Types of bioremediation

For the better understanding of bioremediation and for convenience of the study it may be divided into following types according to biological agents used for the treatment of toxicants:

4.1. Phytoremediation

In this method plants are used to remove pollutants from the environment. Phytoremediation targets include contaminating metals, metalloids, petroleum hydrocarbons, pesticides etc. In comparison to conventional purification technologies, phytoremediation is a cost effective one. This technology is the main driving force of the researches done in this area resulting into commercialization of the technology. Commercial phytoremediation systems for clean-up of shallow aquifers and water borne contaminants are now in the market. Today, we know about the plants which purify air by absorbing toxic gases and

metals by the application of biotechnology (Li and Li, 2017). Such types of transgenic plants are being developed which can remove more and more contaminants from the environment. Sometimes it is also referred to as 'green clean' which means *cleaning up of environment with the help of plants*. Some plants, most notably, the Chinese brake fern (*Pteris vittata*) has been reported to be suitable for arsenic phytoremediation (Alkorta *et al.*, 2004). An American patent registered in 1994 describes how genetically altered members of the family Brassicaceae (family of scavengers) like *Brassica juncea* have shown tremendous potential for clean-up of polluting metals through their roots. The plants accumulate metals to levels between 30 and 1000 times higher than their concentration in the surrounding soil. The metals absorbed by various members of the *Brassica* plant family include antimony, arsenic, barium, beryllium, cadmium, cesium, chromium, cobalt, copper, gold, both stable and unstable form of lead, manganese, mercury, molybdenum, nickel, palladium, plutonium, selenium, silver, strontium, uranium, vanadium, zinc etc.

Dust is a major air pollutant and around 40% of total air pollution in India is contributed by dust pollution. Based on extensive field surveys and experimental studies, the following species have been recommended by NBRI, Lucknow for raising green belts around industrial and urban areas to reduce the dust load: *Ipomoea fistulosa*, *Peltophorum pterocarpum*, *Tectona grandis*, *Ficus bengalensis*, *F. infectoria*, *Terminalia arjuna*.

Following are examples of some plants tolerant to gaseous pollutants:

Plants tolerant to SO₂: *Polyalthia longifolia*, *Terminalia arjuna*, *Acer platanoides*, *Thuja orientalis*.

Plants tolerant to Ozone (Bowler and Fluhr, 2000): *Zinnia elegans*, *Gladiolus spp.*, *Pelargonium graveolens*.

Plants tolerant to NO_x: *Carrisa carandas*, *Codiaeum variegatum*.

Plants tolerant to PAN: *Acer platanoides*, *Chrysanthemum spp.*

4.2. Rhizofiltration

In this form of phytoremediation roots of plants act as *biofilters* i.e. these absorb toxic metals from the contaminated water and accumulate them. Therefore, the water which passes through root zone becomes free from pollutants (Macek *et al.*, 2004). Later, the contaminants are removed from the system by harvesting root biomass. Members of family *Brassicaceae* (the family of scavengers) have shown tremendous potential for the cleanup of polluting metals. The plants roots accumulate metals from the surrounding soil giving a metal content as high as 30% of the dry weight of the plant roots.

Alan Baker, a geobotanist from University of Sheffield, England, discovered a tree *Sebertia acuminata* (the nickel lover) which hyper-accumulates Ni so much that when slashed, it bleeds a jade green liquid. The tree is a native of New Caledonia and accumulates Ni as high as 20% of its dry body weight. Besides, *Acedium* plant absorbs Vanadium and some plants of family *papilionaceae* (e.g. Pea) are known to absorb higher amounts of Molybdenum from soil.

4.3. Microbial bioremediation

Microorganisms like bacteria are proving very important tools for the removal of pollutants from the environment and are thus continuously doing their job of detoxification, tirelessly, with or without coming into the attention of man, who is the only culprit of dumping more and more pollutants into the environment. In the recent past, microbes were first used to treat industrial wastewater as early as 1930s. There was a significant movement in the field of microbial bioremediation when Dr. Howard Worner (USA) first discovered phenol degrading microbes, when began

his research in this field in the early 1950s.

In another breakthrough, Dr. Ananda Mohan Chakrabarty of the General Electric Company (USA) developed a genetically engineered oil eating bacterium (*Pseudomonas putida*). The patent was registered to him in 1980s. It was welcomed by the scientists' community as an answer to pollution problem. The Environment Protection Agency (EPA) reported that bioremediation eliminated both soil and water borne oil contamination at about 1/5th cost of previous method. Since then, bioremediation has been increasingly used to clean up oil pollution in United States and in other countries. In 1989, oil spilled from the Exxon Valdez tanker off the coast of Alaska where these oil eaters helped in clean-up of oil. They degraded the oil efficiently trapped between rocks and under gravel beaches where all other means had failed.

4.4. Zooremediation

It is the process of decontamination of polluted environment by using animals as bioagents. Animals so far used for bioremediation purposes are fish, different arthropods and other filter feeders in aquatic systems and earthworms in solid organic waste management systems. Use of animals in bioremediation is not very encouraging except earthworms because there are so many limitations with them e.g. fish and arthropods may bioaccumulate the toxic compounds and metals which may be biomagnified in the food chain creating many problems to the environment (Gifford *et al.*, 2007).

Solid organic waste generation is a major problem of cities which are facing the threat of being overrun by garbage and the piled up waste and is adversely threatening our health, environment and wellbeing. In this context, vermicomposting - waste degradation through earthworms has proved to be very

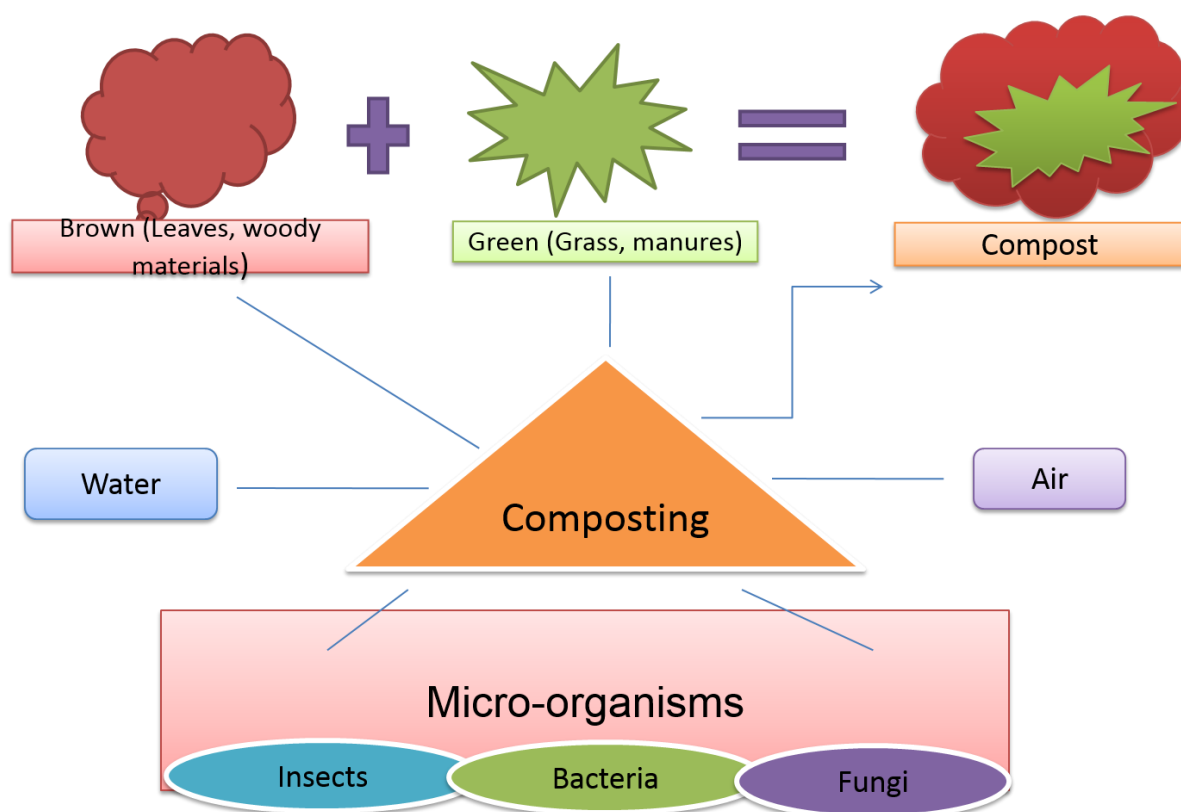


Figure 2: *Ex-situ* bioremediation by composting.

promising. The principles behind this are relatively simple and related to those involved in traditional composting (Figure 2 summarizes composting as a basic *Ex-situ* bioremediation process). In general, vermicomposting consists of 4 major phases: Phase I: Collection of the waste, separation of metal, glass, ceramics etc. from the organic waste, and storage of the organic waste.

Phase II: Earthworm beds are maintained and the earthworms are fed with the organic waste.

Phase III: After the organic waste has been worked over by the earthworms, the vermicompost, cocoons, earthworms and the undigested material are separated.

Phase IV: Packaging of the vermicompost and reintroduction of undigested material into the vermipits.

Certain species of earthworms (*Eisenia fetida*, *E. Andrei*, *Lumbricus rubellus*, *L. hortensis*, *L. terrestris* etc.) can consume organic residues very rapidly and fragment them into much finer particles by passing them through a

grinding gizzard, an organ that all earthworms possess. The earthworms derive their nourishment from the microorganisms that grow upon the organic materials. At the same time they promote further microbial activity in the residues so that the faecal matter or casts that they produce are much more fragmented. During this process, the important plant nutrients in the organic material particularly nitrogen, phosphorus, potassium and calcium are released and converted into forms that are much more soluble and available to plants than those in the parent compounds. Worms can digest waste several times their own weight each day.

4.5. Bioventing

Bioventing is a process of stimulating the natural in situ biodegradation of contaminants in soil by providing air or oxygen to existing soil microorganisms. Bioventing uses low air flow rates to provide only enough oxygen to sustain microbial activity in the vadose zone (Hinchey, 1994). This is an on-site

or *in-situ* bioremediation option for reducing or eliminating contaminants in soil and water. With benefits that include minimal site disturbance and lower cost compared to other remediation technologies, *in-situ* bioremediation continues to be researched and applied with the goal of helping 'close out' specific sites, that is, reducing toxins to safe and/or legally acceptable levels (Agency, 1995, Gibbs *et al.*, 1999).

Among the most promising of these technologies is soil bioventing, the process of supplying oxygen to contaminated soil in hopes of stimulating microbial degradation of contaminants. A typical bioventing setup is appealingly simple: a blower or compressor connected to one or more air-supply wells and a series of soil-gas monitoring wells (Sellers, 1999). The technology of choice for remediating many petroleum wastes, bioventing may eventually be used to treat a wider variety of more recalcitrant toxins (McCauley, 1999a). Bioventing has noteworthy remediation relatives, with distinct principles, goals and applications. Air sparging forces compress air into saturated soil in hope of promoting biodegradation. Unlike sparging, bioventing uses low-pressure air and is generally focused on the vadose or unsaturated zone of soil (McCauley, 1999b). Bioslurping combines bioventing and direct vacuum extraction of contaminants. Soil vapor extraction or soil vacuum extraction (SVE) maximizes volatilization of contaminants and sucks them out of the soil. Bioventing began to mature as a technology after 1988, when researchers on a SVE operation at Hill Air Force Base, Utah, concluded that a significant proportion of contaminant decrease was not due to volatilization, but biodegradation (Agency, 1995; Litchfield, 1993).

4.6. Bioleaching

This principle of bioleaching involves the use of living organisms like microbes in the metal extraction from

their ores. It is one of several applications within biohydrometallurgy and several methods are used to recover Cu, Zn, Pb, As, Ni, Mo, Au, Ag, and Co. Heterotrophic bacteria are widely used in the study of bacterial leaching of manganese from manganese dioxide ores and glucose or other organic compounds are used as a source of energy, rendering their commercial utilization uneconomic (Cornu *et al.* 2017). Coal, especially brown coal from certain coal mines may contain a certain amount of rare metals, such as Germanium (Ge) and Gallium (Ga). The conventional process to recover Ge from brown coal is a lengthy process involving many steps, i.e. burning of the brown coal, recovering of Ge from ashes by sulphuric acid leaching, precipitating of Ge with tannin, roasting of Ge-containing tannin to produce Ge concentrate with the grade of 11%. This process is complex and has a low recovery of 60%, which is sure to bring about a great waste of resource. Instead, a novel process to recover Ge from brown coal in the presence of microorganism has been developed where a germanium recovery of up to 85.33% has been achieved.

4.7. Landfarming

Landfarming is a process in which the soil is excavated and mechanically separated via sieving. In land farming, which is performed in the upper soil zone or in biotreatment cells, contaminated soils are mixed with soil amendments such as soil bulking agents and nutrients and then they are tilled into the earth. Contaminated soils, sediments, or sludges are incorporated into the soil surface and periodically turned over or tilled to aerate the mixture. The material is periodically tilled for aeration. Contaminants are degraded, transformed, and immobilized by microbiological processes and by oxidation. Soil conditions are controlled to optimize the rate of contaminant degradation (Datta *et al.*, 2016). Moisture content, frequency of aeration, and pH are

all conditions that may be controlled. Land Farming differs from composting because it actually incorporates contaminated soil into soil that is uncontaminated. Landfarming is most successful in removing polycyclic aromatic hydrocarbons (PAH) and pentachlorophenol (PCP).

4.8. Bioreactor

Bioreactors treat contaminated soils in both solid and liquid (slurry) phases. The solid phase treatment process mechanically decomposes the soil by attrition and mixing in a closed container. The objective of the mixing is to guarantee that the pollutants, water, air, nutrients, and microorganisms are in permanent contact. An acid or alkalinity may also be added to control the pH (van Deuren and Lloyd, 2002). In fixed bed reactors, compost are added and significantly increase the degradation rate. In rotating drum reactors, the drum has a screw like mechanism in the middle of it that rotates to mix and transport the soil. The liquid phase treatment process uses suspension bioreactors and treats as slurry. The slurry feed enters the system and is rinsed through a vibrating screen to remove debris. Sand is then removed using a sieve or hydrocyclone. If a hydrocyclone is used to remove the sand, the sand falls to the bottom of the cyclone and the fines remain on top. The fines are then treated in a bioreactor. After the treatment, the slurry must be dewatered and the water is then treated with standard wastewater techniques (Kleijntjens and Luyben, 2000).

4.9. Biopiling

It is an *in situ* process that is also known as the heap technique. The first step in the biopiling process is to perform laboratory tests that will determine the biological degradation capabilities of the soil sample. The next step involves the mechanical separation of the soil, which will homogenize the sample and remove any disruptive material such as plastics,

metals, and stones. The stones will then be crushed into smaller pieces and then depending on the degree of contamination will either be added to a pile or sent out for reuse. The soil is then homogenized, meaning that the pollution concentration is averaged out across the entire soil sample. Homogenization allows for biopiling to be more effective (Schulz–Berendt, 2000). Once the soil is piled, nutrients, microbes, oxygen, and substrate are added to start the biological degradation of the contaminants. The results of the initial laboratory tests indicate to the operators which substrates such as bark, lime, or composts needs to be added to the soil. Nutrients such as mineral fertilizers may also be added. Additionally, microorganisms such as fungi, bacteria, or enzymes could be added (Schulz-Berendt, 2000).

4.10. Bio-stimulation

Bio stimulation could be perceived as including the introduction of adequate amounts of water, nutrients, and oxygen into the soil, in order to enhance the activity of indigenous microbial degraders. The concept of bio stimulation is to boost the intrinsic degradation potential of a polluted matrix through the accumulation of amendments, nutrients, or other limiting factors and has been used for a wide variety of xenobiotics (Kadian *et al.*, 2008). Microorganisms do extremely well in thriving on herbicide compounds in the soil by utilizing them as a supply of nutrients and energy. Many herbicides serve as good carbon and/or nitrogen sources for soil microorganism (Qiu *et al.*, 2009). Evidence for their remarkable range of degradative abilities can be seen in the recycling rather than accumulation of vast quantities of biological materials that have been produced throughout the history of life on earth (Dua *et al.*, 2002).

4.11. Bio-augmentation

Bio augmentation is the enhancement of biodegradation of waste

and contaminants in the media by the introduction of adapted competent microbes and nutrients. Microorganisms from *Geobacteraceae* family due to their physiological characteristics can play an important role in the bioremediation of subsurface environments contaminated with organic or metal contaminants (Lovley *et al.*, 2004). In some instances, the rate of biological degradation can be increased through the addition of microorganisms that have been shown to degrade the contaminants of concern at high rates or are particularly well suited to remain active under prevailing site conditions. This process is referred to as bio augmentation. This can be useful if the contaminants are particularly recalcitrant to degradation or if site conditions are extreme (for example: high concentrations or toxicity of contaminants). To be effective, the introduced organism(s) must become distributed throughout the contaminated matrix and compete with the indigenous microorganisms for available nutrients. If they are not distributed throughout the matrix the positive effect will be localized. On the other hand if the introduced organisms compete poorly, they will not persist and the treatment effect will be short lived. The problems encountered using this approach include biofouling of equipment, injection wells and seepage beds. Adjustments to the system, such as the use of new discharge areas, may be required to prevent this from occurring. This approach to bioremediation must be evaluated on a site specific basis.

4.12. Intrinsic bioremediation

Often bioremediation can be accomplished without human intervention by microorganisms that are naturally found in the contaminated matrix. For this approach to be used, it is usually necessary for the rate of contaminant degradation to exceed the rate of contaminant migration. Knowledge of the

following key site characteristics are required to evaluate the likely success of intrinsic remediation; the bioavailability of contaminants, levels of nutrients, the presence of minerals to buffer the pH of the matrix, adequate levels of electron acceptors (either oxygen, nitrate, ferric iron, or sulphate), and site specific contamination migration rates. This approach deals with stimulation of indigenous or naturally occurring microbial populations by feeding them nutrients and oxygen to increase their metabolic activity.

5. Microbes involved in bioremediation

Microorganisms are responsible for biodegradation in various diverse environmental conditions. These microorganisms include: *Acinethobacter*, *Actinobacter*, *Acaligenes*, *Arthrobacter*, *Bacillins*, *Berijerinckia*, *Flavobacterium*, *Methylosinus*, *Mycrobacterium*, *Mycococcus*, *Nitrosomonas*, *Nocardia*, *Penicillium*, *Phanerochaete*, *Pseudomonas*, *Rhizoctomia*, *Serratia*, *Trametes* and *Xanthofacter*. Individual microorganisms are not efficient in mineralization of harmful substances. Thorough mineralization results in a progressive degradation by a group of microorganisms (or microbial consortiums) and involves coaction and co-metabolism actions. Microorganisms in various habitats have remarkable physiological flexibility, so they are able to make use of and often mineralize an enormous number of organic molecules. Several other requirements for microbial growth in biodegradation process are listed in Table 2. Some microbes with specific biodegradation capabilities are discussed below.

Pseudomonas putida: In context of bioremediation, it is a microorganism found in farmland soil involving high impact xenobiotics including organophosphate insecticides, petroleum hydrocarbons, and both monocyclic and

Table 2: Requirements for microbial growth in bioremediation process (Source: Vidali, 2001)

Requirement	Description
Nutrients	The growth and activity of the microorganisms must be estimated by adequate maintenance and supply of nutrients. These nutrients are the basic building blocks of life and allow microbes to create the necessary enzymes to break down the contaminants. Bio-stimulation usually involves the addition of nutrients and oxygen to help native microorganisms. Nitrogen (ammonic, nitrate, or organic nitrogen) and phosphorous (orthophosphate or organic phosphorous) are commonly used as the limiting nutrients. In certain anaerobic systems, the use of trace metals (e.g. iron, nickel, cobalt, molybdenum and zinc) is generally preferred.
Carbon source	Carbon which is considered as the most basic element of living forms is required in larger quantities than other elements. Carbon contained in many organic contaminants may function as a carbon source for cell growth. If the organism involved is an autotroph CO_2 or HCO_3^- in solution is required. In some cases, contaminant levels are too low to supply suitable levels of carbon to cell. In these cases the addition of carbon sources may be required.
Electron acceptor	All respiring bacteria require a terminal electron acceptor. In some cases, the organic contaminant may serve in this capacity. The most common electron acceptor in aerobic bioremediation processes is dissolved oxygen. Under anaerobic conditions, NO_3^- , SO_4^{2-} , Fe^{3+} , and CO_2 may serve as terminal electron acceptors. Certain co-metabolic changes are carried out by fermentative and other anaerobic organisms, in which terminal electron acceptors are not necessary.
Energy source	In the case of primary metabolism, the organic contaminant supplies energy required for growth. This is not the case when the contaminant is metabolized via secondary metabolism or co-metabolism or as a terminal electron acceptor. If the contaminant does not serve as a source of energy, the addition of a primary substrate(s) is required.
Soil moisture	Microbial growth and activity is also affected by moisture content. The water-holding capacity suggested for bioremediation process may range from 25% – 28%.
Temperature	Temperature regulates the rates of growth and metabolic activity. Surface soils are particularly susceptible to wide variations in temperature. Generally, mesophilic conditions are appropriate for most applications (with composting being a notable exception).
pH	A pH is another important factor that affects bioremediation process. If the soil is acidic, it is possible to raise pH by adding lime. A pH fluctuating between 6.5 and 7.5 is generally considered optimal. The pH of most ground water (8.0–8.5) is not considered inhibitory.
Absence of toxic metals	Many contaminated sites contain a mixture of chemicals, organic and inorganic, which may be inhibitory or toxic to microorganisms. Heavy metals and phenolic compounds are of particular concerns.
Adequate contact between microorganisms and substrates	For contaminants to be available for microbial uptake it must be present in aqueous phase. Thus contaminants that exist as non-aqueous phase liquids or are sequestered within a solid phase may not be readily metabolized. For degradation it is necessary that bacteria and the contaminants be in contact. This is not easily achieved, as neither the microbes nor contaminants are uniformly spread in the soil. It is possible to develop the mobilization of the contaminant utilizing some surfactants such as sodium dodecyl sulphate (SDS).
Time	Time is an important factor in the start-up of bioremediation systems. Even the above mentioned parameters are met, lag phases are often observed prior to the onset of activity. In some cases, the intense bacterial population shifts that are required for bioremediation will increase periods of slow activity.

polycyclic aromatics (Iyer and Damania, 2016).

Dechloromonas aromatic: This bacterium is involved in degradation of aromatic compounds like benzene in nitrate reducing conditions as well as physiological and molecular characterization in anaerobic mixed cultures (Ulrich and Edwards, 2003).

Deinococcus radiodurans – In field of development of bioremediation strategies, this bacterium plays a role as a radiation resistant organism. It is used for the treatment of mixed radioactive wastes containing ionic mercury (Brim et al., 2000). The radioactive waste sites can be treated by this strategy of bioremediation.

Methylibium petroleiphilum – Also known as PM1 strain that is involved in methyl tert butyl ether (MTBE) bioremediation. MTBE is degraded by this strain using the contaminants as source of carbon and energy (Hanson et al., 1999).

Alcanivorax borkumensis is a rod-shaped bacterium having capability of consuming hydrocarbons and produces carbon dioxide. Hence it can be used readily in oil damaged environment (Santisi et al., 2015).

Phanerochaete chrysosporium – It is the first fungi involved in degradation of organic pollutants (Kadri et al., 2017).

6. Genetically modified organisms

Bioremediation by means of microorganisms is not significant for treatment of all types of pollutants. For example, heavy metals such as cadmium and lead are not freely absorbed or taken by organisms. The role of genetically modified organisms in the process of bioremediation has emerged as a new tool (Jafari et al., 2013). A genetically modified organism, or GMO, is an organism that has an altered DNA configuration made through genetic engineering. Most of the genetically modified organisms have been transformed with DNA from other

organisms like bacteria, plant, virus or animal and thus these are also referred to as transgenic organisms (Ozcan et al., 2012).

6.1. Role of GMOs in environmental management

Genetically modified organisms can be used to clean up the environment by bioremediation. Effects of some genetically modified microorganisms are unstable and vary according to species, changes in population structure and loss of some functions, to the formation of toxic metabolites. Presence of high and active microorganisms makes the process of bioremediation more operative and they must adapt with the changing environmental conditions. *Deinococcus radiodurans* that exhibit toluene dioxygenase to clear-out toxic elements that are found in radioactive waste sites was used by Lange (1998) as a recombinant. *Deinococcus radiodurans* is known to have two properties, first it is resistant to radiation and secondly it can degrade chlorobenzene in radioactive environments (Lange et al., 1998). Then again, it can only be produced in an environment at temperatures less than 39°C and as radioactive sites generally have high temperatures, so a bacterium is required that can function at higher temperatures. Another well-known example for the application of GMOs in the management of environmental issues can be cited through certain bacteria that can yield biodegradable plastics and this quality of bacteria were transferred to microbes which were cultured in the laboratory and now a days they have enabled the wide scale greening of plastic industry.

In the early 1990s, Zeneca, a British company, established a microbially manufactured biodegradable plastic called Biopol (polyhydroxyalkanoate, or PHA). The plastic was made using a GM bacterium, *Ralstonia eutropha*, to transform glucose and a variety of organic acids into a

flexible polymer (Perez-Pantoja *et al.*, 2008). GMOs which are able to metabolize oil and heavy metals through their bacterially encoded ability may prove effective for the bioremediation process. Simultaneously, genetically engineered microorganisms (GEMs) have shown possible uses for bioremediation in soil, groundwater, and activated sludge environments, due to the enriched degradative capabilities for extensive range of contaminants. Recent advances in molecular biology have unlocked new perceptions for the development of engineering microorganisms with the purpose of performing specific bioremediation.

From the biological safety view it has also been reported that not all naturally occurring bacteria are ideal as bioremediation agents. For instance, *Burkholderia cepacia* would be both used as an agent for bioremediation and for biological regulator of phytopathogens. However, it causes cystic fibrosis in humans and it is also found to be resistant to many antibiotics (Holmes *et al.*, 1998). For these reasons, the US Environmental Protection Agency (EPA) has led to its elimination to be used as an environmental agent (Davison, 2005).

7. Types of pollution controlled by bioremediation

The population explosion throughout the world has led to an increase in the polluted soil and water regions. As the number of people continues increasing day by day it also results in the overuse of natural resource like air, water and land resources. For these reasons, there occurs rapid expansion of industries, food, health care, vehicles, etc. but it is very challenging to retain the quality of life with all these new expansions, which are critical to the environment in which we live. Since the quality of life is very much linked to the overall quality of the environment, worldwide measures are taken to sustain and preserve the

environment. Bioremediation is one of the emerging biological strategies which is applicable to the repair of damaged environment.

The three main types of pollution (Soil, water and marine pollution) that are controlled by bioremediation using a variety of microorganisms which belong to different environments and are active members of microbial associations are discussed here.

7.1. Marine pollution

The derivatives of petroleum are the most important source of energy for industry and societies. The probable cause of oil spills in marine environment is mainly through the frequent transport of petroleum across the world. Moreover, it is broadly known that petroleum hydrocarbons pollution has obstructed, and spoiled the world oceans, seas and coastal zones and due to this, the Earth's health sustainability is at high risk. In marine environment too, bioremediation is considered as an economic and ecological biotechnology tool for the handling of polluted wastes (Paniagua-Michel and Rosales, 2015). The frequently applied bioremediation methods that can be used in marine environments facing disturbance due to oil spills are (i) using the process of bio augmentation by the addition of oil degrading bacteria so as to grow or improve the existing bacterial biota, and (ii) use of composts (nutrients), to encourage and stimulate the growth of native oil degraders, which is called bio-stimulation. In the case of oil spills, the processes make use of the catabolic skill of microorganism feeding on oil. Several workers (Odu, 1978; Sloan, 1987; Ijah and Antai, 1988; Okpokwasili and Okorie, 1988; Barnhart and Meyers, 1989; Anon, 1990; Pritchard, 1991; Pritchard and Costa, 1991; Hoyle, 1992; Ijah, 2002; and Ijah, 2003) have pronounced numerous application of microorganism in the bioremediation of oil pollution with promising results.

7.2. Water pollution

Water pollution is a subject of great global concern, and it can be largely distributed into three main groups, that is, contamination by organic compounds, inorganic compounds (e.g., heavy metals), and microorganisms. It has caused an irreparable damage to aquatic ecosystems through environmental contamination by heavy metals from anthropogenic and industrial activities. Other sources of heavy metals comprise the mining and smelting of ores, run-off from storage batteries and automobile exhaust, and the manufacturing and inadequate use of fertilizers, pesticides, and many others. The bioremediation approach works on the high metal binding ability of biological agents, which can eliminate heavy metals from polluted sites with high efficacy. Specimens of microorganisms studied and advantageously used in bioremediation treatments for heavy metals include the following: (i) Bacteria: *Arthrobacter spp.*, *Pseudomonas veronii*, *Burkholderia spp.*, *Kocuria flava*, *Bacillus cereus*, and *Sporosarcina ginsengisoli* (Gautam et al., 2011; Cycon et al., 2017); (ii) fungi: *Penicillium canescens*, *Aspergillus versicolor*, and *Aspergillus fumigatus*; (iii) yeast: *Saccharomyces cerevisiae* and *Candida utilis*; (iv) algae: *Cladophora fascicularis*, *Spirogyra spp.* and *Cladophora spp.*, and *Spirogyra spp.* and *Spirullina spp.*

7.3. Soil pollution

Decontamination of soil can be processed through both *ex situ* and *in situ* remediation techniques. *Ex situ* thermal remediation processes are best for use for the following contaminants: petroleum hydrocarbons (TPH), polycyclic aromatic hydrocarbons (PAH), benzene, toluene, ethylbenzene, xylenes (BTEX), phenolic compounds, cyanides, and chlorinated compounds like polychlorinated biphenyls (PCB), pentachlorophenol (PCP), chlorinated hydrocarbons, chlorinated pesticides, polychlorinated

dibenzodioxins (PCDD), and polychlorinated dibenzofurans (PCDF) (Koning et al., 2000). The biological processes of *ex situ* remediation involve: composting, landfarming, biopiling and the use of bioreactors. Alternatively, bioventing, biosparging, bioslurping and phytoremediation along with physical, chemical, and thermal processes are included in *in situ* remediation techniques for treating soil pollution.

8. Advantages of bioremediation

For successful bioremediation to occur, the bioremediation methods rest on having the right microbes in the right place with the right environmental factors for the process of degradation. Bioremediation is considered more advantageous over conventional techniques like land filling or incineration. Microbes capable of destroying the contaminants increase in number when the pollutant is present and when the pollutant is degraded, the biodegradative population declines. The remains for the treatment are generally harmless products and include carbon dioxide, water and cell biomass. Theoretically, bioremediation is useful for the thorough damage of a wide variety of contaminants. Many compounds that are officially considered to be unsafe can be transformed to nontoxic products. Bioremediation also reduces the chance of future problem associated with treatment and disposal of polluted waste (Rajwade et al., 2015). Bioremediation that is performed on site is often less expensive and site interruption is nominal, it removes waste permanently, reduces long-term problem, and has better public acceptance, with regulatory encouragement, and it can be tied with other physical or chemical treatment methods.

9. Disadvantages of bioremediation

There are limitations to every process and so is bioremediation which is limited to those compounds that are biodegradable. It has been found that compounds such as heavy metals, radionuclides and some chlorinated compounds are not prone to rapid and complete degradation through bioremediation and there are cases where microbial breakdown of contaminants has resulted in toxic metabolites. Like most of the biological processes, bioremediation is also highly specific. The site factors that are significant and required for the success include the presence of metabolically proficient microbial populations, proper environmental growth conditions, and appropriate levels of nutrients and waste products (Tran *et al.*, 2015). Bioremediation is logically a thorough procedure which can be designed for site-specific conditions, i.e. before proceeding to cleaning of the sites, one has to do treatment studies on a minor scale (Ramrakhiani *et al.*, 2016). The process of bioremediation often involves the time factor as it takes much more time than other treatment options, such as diggings and removal of soil or incineration. A second drawback to this technique in contrast to other remediation techniques is its relative sensitivity to environmental factors for example temperature, pH, and the presence of various other substances or organisms. There are many questions that should be answered before using bioremediation: Whether the contaminant is biodegradable? Is biodegradation occurring in the natural site? Are environmental conditions appropriate for biodegradation? Where the waste will be disposed if it is not degraded completely? These questions can be answered by doing site classification and also by treatability studies.

10. A current update on bioremediation

The existing bioremediation strategies are based upon either

absorption or metabolism of the polluting compound. If the xenobiotic compound acts as a source of energy, carbon or any nutrients then it is absorbed by the bioremediators. Otherwise, it is co-metabolised by a single organism or a group of different organisms' together acting as bioremediators. To enhance bioremediation by these strategies an adequate understanding of microbial behaviour is of prime importance (Alvarez *et al.*, 2017). Furthermore, advanced engineering techniques have been developed to stimulate the microorganism involved in detoxification process. For instance, sparging of gaseous phase by enhanced mechanisms has led to complete utilization of bioremediation potential of several aerobic microbes. Research is also being carried out to promote the availability of pollutants to the microbes. Advance techniques such as use of surfactants, solubilisation of pollutants by exposure to steam, heat or heated water, and application of high pressure are being used for this purpose (Rittmann, 1993; Shukla *et al.*, 2014).

The approach of developing designer microbes (GMOs) with the help of recombinant DNA technology has already being discussed in detail. Several other innovative technologies such as transcriptome and proteome analysis, molecular profiling, pyro sequencing, metatranscriptomics and metaproteomics, mass spectrometry, microarrays, and numerous bioinformatics applications are helping in realization of complete potential of microbes for bioremediation (Kulshreshtha, 2012; Rittmann, 1993).

In another very innovative approach the toxic industrial, domestic and agricultural waste can be converted into useful forms and products such as bioethanol, biogas, biofuels, single cell proteins etc., (Kulshreshtha, 2012). Recently, immobilization of microbial cells or the enzymes released by them through several mechanisms such as adsorption, electrostatic binding, covalent binding, aggregation, crosslinking,

entrapment, and encapsulation has been used extensively for bioremediation. Such immobilization of microbes improves the bioremediation process by facilitating reuse of microbes or the catalyst involved, and also reduce the costs of the process (Dzionek *et al.*, 2016).

11. Bioremediation and sustainability

Sustainable remediation technology has been defined as the cautious use of natural resources by using a combination of remedies which maximizes the net benefit on human health and environment. Environmental modification through bioremediation is a dominant part of bio economy and sustainable development. In recent years, there has been an increase in the use of biodiversity as raw material for environmental decontamination. On the other hand volume and diversity of contaminated substrates (water, soil and air) are increasing due to anthropogenic and technogenic sources. Microorganisms have occupied some of the most life-threatening environments on the earth and some of them are capable of degrading the pollutants that are produced through our industries. Ecological engineering has been suggested as a theoretical framework to project “sustainable ecosystems that incorporate human society with its natural environment for the profit of both”. Energy use is one of the most important sustainability concerns for conventional remediation projects. *Ex-situ* remediation is typically too energy intensive to be considered ecological engineering. In sustainable bioremediation external energy input is preferably used only in the initiation phase to start a process that is later driven by solar energy and the exemplified chemical energy of the pollutant itself. The engineer’s role is to help provide the proper conditions in which such a process can take place. Since the objective of bioremediation projects is to eliminate pollution that employs stress on the ecosystem, it

involves an ecosystem conservation approach. However, if large amounts of soil are physically removed from the site in *ex-situ* operations the remediation itself might be a threat to the ecosystem. In view of economic sustainability, a number of organic by-products are used which include lignocellulosic wastes such as sugarcane, bagasse and sawdust, crop residues such as coffee pulp and molasses and whey, a by-product of the dairy industry. These are also identified to increase the degradation of diverse toxic compounds. A good bioremediation methodology will include the planned use of all native microbes in an engineered way to accomplish the best possible purification levels. In summary, we can conclude that although bioremediation appears to be a promising alternative for the remediation of contaminants in different ecosystems and is also contributing to sustainability of the environment but it is still in the developmental phase.

12. Future perspectives

The application of microorganisms to increase the fertility of soil conditions and removing the soil contaminations through bioremediation technology is extensively used in Europe and USA. In Asia particularly in India as major agriculture dependent country, progress has been made in applying microorganisms to the restoration of polluted soil through bioremediation processes. However, the application of bioremediation technology in the restoration of ecosystem and soil management is used less compared to Europe and USA. Hence, extensive research programs are needed to increase the capabilities of bioremediation to deep, extensive, subsurface contamination due to chlorinated hydrocarbons and complex mixed wastes, including soils and groundwater. Besides that, The American Academy of Microbiology (AAM) has concluded that enough knowledge is now

available for field trials of bioremediation technology for organic compounds and further they emphasized that research is needed for the following classes of environmental pollutants: metals, metalloids, radionuclides and complex polycyclic hydrocarbons. The on-going microbial genomics studies will deliver more robust technologies for the bioremediation of metal – contaminated waters and land. Exciting developments in the use of microorganisms for the recycling of metal waste, with the formation of novel biominerals with unique properties are also predicted in the near future. Moreover, a wide diversity of microbes with detoxification abilities is waiting to be explored. The inadequate knowledge about microbes and their natural role in the environment could affect the acceptability of their uses. The understanding of the diversity of microbial community's in petroleum contaminated environment is essential to get a better insight into potential oil degraders and to understand their genetics and biochemistry that will result in developing appropriate bioremediation strategies, thus, preserving the long-term sustainability of natural terrestrial and marine ecosystems.

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Sea Urchin - A New Potential Marine Bio-resource for Human Health

M. Aminur Rahman^{1,*}, Fatimah Md. Yusoff^{1,2}, Kasi Marimuthu³ and Yuji Arakaki⁴

¹Laboratory of Marine Biotechnology, Institute of Bioscience, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia; ²Department of Aquaculture, Faculty of Agriculture, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia; ³Department of Biotechnology, Faculty of Applied Sciences, AIMST University, 08100 Bedong, Kedah Darul Aman, Malaysia; ⁴Department of Tourism, Faculty of International Studies, Meio University, Nago, Okinawa-905-8585, Japan;

*Correspondence: aminur1963@gmail.com; Tel: +60 3-8947-2141

Abstract: Sea urchin gonads usually called as “Sea urchin Roe” or “Uni”, are important food delicacies in different parts of the world. In Asian, Mediterranean and Western Hemisphere countries, the roe of sea urchins is considered as a highly prized delicacy sea food because of its tastes and also have long been utilized as extravagance foods in Japan. Peoples of the Asian Pacific region have long been utilizing it for improving general body tone and also treatment for a number of diseases. It has been reported that, sea urchin gonads are found to be rich with high-quantities of bioactive compounds, such as polyunsaturated fatty acids (PUFAs) and β -carotenes. The PUFAs, particularly eicosapentaenoic acid (EPA, C20:5 (n-3)) and docosahexanoic acid (DHA C22:6 (n-3)), have profound significant effects on arrhythmia, cardiovascular diseases and cancer. β -carotene and some xanthophylls have strong pro-vitamin activity and can be used to prevent tumor development and light sensibility. The sea urchin fisheries in recent years have extended so impressively that the natural populations of them have been overexploited to meet-up the increasing demand. Not surprisingly, the continued high demand and the decrease in supply have headed towards a pronounced interest for the commercial aquaculture of sea urchins. Global sea urchin harvesting, having peaks at 120,000 metric tons in 1995, are presently in the scale of around 82,000 metric tons. However, these declining arrays evidently mirror the overfishing of major fishery grounds and focus the necessity for conservation measures, aquaculture development and sustainable fisheries management. Once the natural stocks decrease, the higher market demand for foodstuff, nutraceuticals, pharmaceuticals and cosmeceuticals, increases the value of the manufactured goods and therefore, culturing seems to become economically feasible. As per this assessment exhibits, there have been intense progresses in the aquaculture protocols of sea urchins during the past 15-20 years, we can come to the end that presently the main impediments to successful farming are actually managerial, cultural, conservational and economical rather than biological and ecological. Expected that demand is implausible to decline, the commercial value of future product will be increased. Hence, the fortune of sea urchin is strictly connected to those fisheries, whose prospect would eventually depend on the stock improvement, aquaculture production, fishery management, roe enhancement and market forces that will play a significant role to give a structure of this expanding industry.

Keywords: Biology; culture; ecology; health; management; roe; sea urchin

1. Introduction

Sea urchins (Echinodermata: Echinoidea) are important marine bio-resources for conducting research in diverse areas of ecology, biology, biodiversity, aquaculture, conservation, taxonomy and evolution. Simultaneously, they are utilized as raw material to produce food-stuff, particularly, the product of processing gonads recognized as “Sea urchin Roe” (Kaneniwa and Takagi, 1986; Oshima et al., 1986; Ichihiro, 1993). It is also one of the highly prized seafood delicacy owing to the high tastes in Asian, Mediterranean and Western Hemisphere countries, for instance, Chile and Barbados (Kaneniwa and Takagi, 1986; Lawrence et al., 1997; Lawrence et al., 1997; Yur'eva et al., 2003; Rahman and Yusoff, 2010; Rahman et al., 2013a, 2014a, b; Parvez et al., 2016a, b). In Japan, sea urchin gonads (either in the state of fresh or processed foods) have long since been consumed as high-quality luxury foods (Shimabukoro, 1991; Rahman et al., 2014a,b, Parvez et al., 2016a,b) and the roe can sell for as much as AU\$450/kg (Richard, 2004). Due to the increasing demands for sea urchins, Japan imports big amounts from USA, South Korea, Thailand and other producers, thus has elevated concerns about overfishing, and hence, making it one of the valuable sea foods in the world (Hagen, 1996; Rahman et al., 2014a; Parvez et al., 2016a,b). Traditionally, sea urchin gonads have long been used by the peoples of the Asian Pacific Region, as a remedy for improving general body tones, treatment for a number of diseases and increasing the sexual potency of middle-aged men (Seifulla et al., 1995; Yur'eva et al., 2003). However, in the recent years, the fisheries of sea urchins have been expanded so highly that the natural population in Chile, Japan, France, Canada and different parts of USA have been overexploited to meet up the great demand (Lawrence et al., 2001; Andrew et al., 2002, 2004; Rahman et al., 2005, 2012a,b, 2013b, 2014b; Parvez et

al., 2016b). Not astonishingly, the continuous strong demand and the decline in supply headed to a pronounced increase in awareness for aquaculture of sea urchins, predominantly, in those parts wherein their natural populations have been dwindled (Lawrence et al., 1997; Lawrence, 2007). The species of sea urchins whose gonads have high commercial values could be obtained from a number of genera such as: *Tripneustes*, *Strongylocentrotus*, *Paracentrotus*, *Loxechinus*, *Echinus*, *Centrostephanus*, *Hemicentrotus*, *Lytechinus*, *Diadema*, *Arbacia*, *Colobocentrotus*, *Anthocidaris*, *Psammechinus*, *Evechinus*, *Heliocidaris*, *Echinometra*, *Toxopneustes*, *Pseudocentrotus* and *Pseudoboletia* (Sloan, 1985; Saito, 1992; Keesing and Hall, 1998; Lawrence, 2007; Rahman et al., 2014b).

Nevertheless, the majority of the sea urchins fisheries have followed the same trends of quick expansion to an unsustainable top, followed by a correspondingly speedy decline. Global sea urchin harvesting reached to 20,000 metric tons in 1995, are presently in the state of around 82,000 metric tons with an alarming decreasing rate of 32% (FAO, 2010; Carboni et al., 2012; Rahman et al., 2014b; Parvez et al., 2016b) (Figure 1). However, the newly extended sea urchin fishery (*Loxechinus albus*) from Chile covers half amounts of the world catch (Rahman et al., 2014b; Parvez et al., 2016b). The other main sea urchin fisheries, landed in tonnage, are in Japan, Maine and California (United States), and British Colombia (Canada) (Andrew et al., 2002). In case of Europe, the commercial sea urchin (*Paracentrotus lividus*) in Ireland and France were overfished to supply the French markets (Barnes et al., 2002). There have been reported the large populations and abundances of edible urchins in Norway (*Strongylocentrotus droebachiensis*) and Scotland (*Echinus esculentus* and *Psammechinus milaris*). However, these stocks are not suitable for profitable fishing because their roe amounts are either very small or too flex-

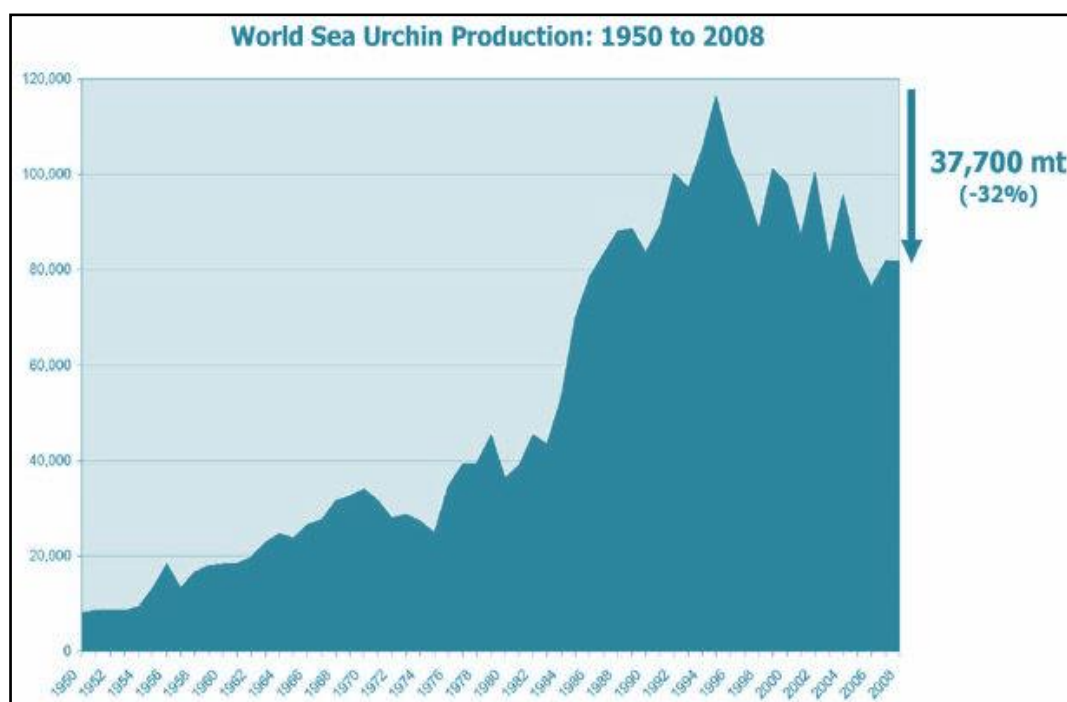


Figure 1: Global production of sea urchin fisheries from 1950 to 2008 (FAO, 2010).

ible (Hagen, 2000; Kelly, 2000, 2005; Sivertsen, 2004; Rahman *et al.*, 2014b). Nonetheless, the continuous declining patterns noticeably reveal the overexploitation of most of the fishery grounds and focus the necessity for proper conservation policies, sustainable management strategies and appropriate aquaculture practices.

2. Biology and ecology of major species

The most parts of the world involved in sea urchin production are linked with main sites of primary productivity. In the subtropical and tropical regions, these are usually associated with seagrass beds, while in the temperate regions, with kelp forests. Urchins commonly occur at lower densities within the kelp communities, overgrazing on kelps and then lead to the establishment of flats dominated by encrusting macroalgae (called as coralline flats and barrens). Both of the communities (barrens and kelp forests) are often found near to each other, making either a mosaic of stable patches or strata.

The biological features of sea urchins vary largely among species. The species

inhabiting in temperate zones mostly have moderate longevity between 10 and 20 years, even though highest longevity of 100 years has been reported. Growth and production performances are flexible and greatly dependent on quality food and nutrition. In conditions with lower density, growth rates are usually high, while at high densities, growth rates are low. However, the density of sea urchins is not the single factor that starts the construction of barrens or flats. Inter-annual events that trigger warming (e.g., El Nino), are known to cause kelp dieback which then leads to overgrazing and the maintenance of barrens in the extended period. The exclusion of predators that would then cause moderate urchin densities may also endorse conditions leading to overgrazing. Usually, densities of sea urchins are the highest in barrens, but individual growth performance and overall productivity is low owing to competition for food and perhaps due to the deficient nourishment.

The most efficient predator of sea urchins, particularly the sea otter and its re-establishment around the North is having main effects on sea urchin fisheries. In areas where populations have been re-

established, predation can far exceed fishing pressures and urchin densities have been reduced to levels below that which is required for fishing to be profitable (Andrew *et al.*, 2002). Recruitment mortality is also affected by different types of habitats. It has been reported that mortality of juveniles among a number of species is higher in kelp forests because of the occurrences of micro-predators in their habitats (Tegner, 2001).

Given the similar habitat types, a number of edible sea urchin species share some similarities in their distribution, abundance and reproduction. Yet, they are differed in their fundamental biological characteristics such as growth, survival, production, maturity and longevity.

2.1. Green sea urchin (*Strongylocentrotus droebachiensis*)

Green sea urchin has a circumpolar distribution and occurs through the North Atlantic to the North Pacific. The fisheries of *S. droebachiensis* have been focused in Maine and the Canadian Maritimes but smaller fisheries are concentrated in Alaska, British Colombia, Washington and Iceland. The species is mostly common in the intertidal zone to a depth of 50 m, where it is closely related with kelp beds (Scheibling and Hatcher, 2001). Two distinct growth rates have been identified, but the overall growth rates are found to be moderate. The fast growing form attains the minimum legal size between 4 and 6 years and can live for 16-20 years. On the other hand, the slow growing one inhabits for 8-12 years and never attains the optimum legal size (Andrew *et al.*, 2002). In British Colombia, the minimum legal size is not more than 55 mm TD (test diameter) and in most cases, the time needed to grow to this size is supposed to range from 4 to 8 years (Taylor 2004). This sea urchin attains sexual maturity within 1-2 years and the first spawning occurs between mid-winters to early spring when it reaches to 45-50 mm in TD.

2.2. Red sea urchin (*Strongylocentrotus franciscanus*)

Strongylocentrotus franciscanus usually referred to as red sea urchin, is the biggest echinoid in the world and commonly occurs along the West Coast of North America, extending from Baja California to the Aleutian Archipelago and the coast of Siberia and northern. The ranges of their habitats encompass northern-wards up the west coast to Sitka and Kodiak AK at 58°N (Tegner, 2001). This species is also occurs in the subtidal zone to a depth of 50 m seawards and is intensely associated with kelp forests. Growth performance in the earlier stage of urchin is comparatively fast and the species shows the highest longevity. TD at recruitment is about 90 mm, which is usually attains in around 6-8 years of the age. The maximum size is around 200 mm and the individuals over 150 mm TD are older than 100 years (Tegner, 2001). Spawning commonly occurs over the spring and summer months when the urchins attain sexual maturity at around 50 mm TD and the spawning usually over summer and spring months.

2.3. Japanese green sea urchin (*Strongylocentrotus intermedius*)

Strongylocentrotus intermedius commonly known as Japanese green sea urchin is the 2nd most economically important regular echinoid in Japan. This species is distributed along the Asian and Siberian coast of the Pacific. This species is common in intertidal shallow waters around Hokkaido and is exploited commercially from Aomori, Irate and Hokkaido (Agatsuma, 2001a). It generally occurs in shallower stony substratum and is usually associated with kelp forests (Agatsuma, 2001a). The matured adult of *S. intermedius* contains small reddish-yellow gonads, which do have a good taste and thus listed on Tsukiji as Japanese. It is well-adapted to cold water and the growth restriction does not appear to be related to the temperature limits (Agatsuma, 2001a). Density and nutrition

are the two major factors influencing growth of urchins. In appropriate culture conditions, the urchin attains 40 mm TD within 2-4 years and the maximum size of 55 mm TD at ages between 6 and 10 years. It obtains sexual maturity at the age of 2 years with the size of 30-35 mm TD and the spawning usually occurs in autumn and spring.

2.4. *Strongylocentrotus nudus*

This species has been recorded on Tsukiji as Japanese and also considered as the most commonly harvested edible sea urchin in Japan and accounts for ~ 44% of the total commercial catch (Agatsuma 2001b). It is found on inter- and sub-tidal rocky bottoms extending from Dalian, China northwards to Primorskyi Kray, Russia and in Japan where it is found in the Pacific from Sagami Bay to Cape Erimo on Hokkaido and in the Sea of Japan from Omi Island in Yamaguchi to Soya Cape northern Hokkaido. The urchins generally reach the legal size (40 mm) in 2 to 4 years when feeding on perennial Laminarians whereas they take 7 to 8 years on coralline flats. It occurs in the intertidal to subtidal rocky reefs and is strongly associated with kelp communities. Juveniles recruit to coralline flats and move to adjacent kelp forests. In kelp forests individuals reach 50 mm TD in 2 to 4 years, whereas 7 to 8 years reported in coralline flats (Agatsuma, 2001b). Maximum longevity is reported as 14 to 15 years. Sexual maturity is attained at 40 to 45 mm TD, and spawning takes place in autumn.

2.5. *Purple sea urchin (Strongylocentrotus purpuratus)*

The sea urchin *S. purpuratus*, usually recognized as purple sea urchin, inhabits along the eastern edge of the Pacific coast of North America, extending from British Columbia, Canada to Ensenada, Mexico. It occurs abundantly in lower intertidal and nearshore subtidal communities but has been found to a maximum depth of 150 m (Tegner, 2001). This species is

closely associated with kelp beds and its growth is extremely flexible and reliant on the availability of algae. The maximum size has been recorded to be 100 mm TD. *S. purpuratus* usually attains sexual maturity around 2 years of age, and spawns during their natural breeding season, extending from January to March.

2.6. *Purple crowned sea urchin (Centrostephanus rodgersii)*

The purple crowned urchin experiences a subtropical distribution throughout the water areas of Australia and New Zealand, but most abundantly occurs in Eastern Australia. This species has also been reported to be extended into Bass Strait and the East Coast of Tasmania (Rdger, 1999) and is possibly related with the warming of coastal waters around the region (Andrew and Byrne, 2001). It is one of the large urchins with long dark purple, black to red spines that have iridescent blue/green sheen. *Centrostephanus rodgersii* is usually seen in large numbers and plays an important ecological role in intertidal near shore rocky reefs by cleaning the areas of kelp. Growth performances follow the medium trends and the individuals attain 70-90 mm TD within the age between 4 and 10 years. The longest size (120 mm TD) achieved when the urchin become 20 years of age. The adult urchin attains sexual maturity at the size between 40 and 60 mm TD and spawning occurs during the winter months.

2.7. *Kina (Evechinus chloroticus)*

Evechinus chloroticus well-known as kina is a sea urchin endemic to New Zealand waters. The distribution of kina is intensely linked within kelp beds or aggregating in nearby barrens. It is typically occurs from the intertidal area to a depth of 14 m; even some are found in 60 m. Growth rate is moderate and the individuals reach to a size of 50 mm within the age of 4 years. However, *E. chloroticus* attains the maximum size of 80-100 mm in 8-9 years of age. Depending on the site, the longevity of this urchin varies be-

tween 10 and 20 years (Barker, 2001). It is predominantly herbivorous, feeding mainly on brown algae, red algae and encrusting substrate (Barker, 2007). Kina usually reaches sexual maturity within 3-4 years of age having a size range between 40 and 50 mm TD and spawns in spring, extending from November to February (Barker, 2007).

2.8. Chilean red sea urchin (*Loxechinus albus*)

The Chilean red sea urchin, *Loxechinus albus*, is one of the reasonably slow-growing urchins, commonly found around the Pacific coasts of South America from Isla Labos de Afuera in Peru to the Southern tip of South and usually occurs within the depths ranged from the intertidal zone to a maximum of depth of 340 m (Vasquez, 2001). This urchin attains sizes up to 130 mm TD and can live until 20 years of age (Andrew et al., 2002). It is considered as one of the important commercial species along the south-west coast of South America due to high taste qualities. The distribution of *L. albus* is mostly on rocky substrates and closely related with the kelp beds. It is an herbivore and seems most likely to feed on whatever species of alga grow nearby. The urchin is comparatively slow-growing, attaining a maximum size of 130 mm TD. Spawning period differs depending upon its destitution patterns; happening in spring to summer in the north, summer in the south and spring in the extreme south.

2.9. Variegated sea urchin (*Lytechinus variegatus*)

The variegated sea urchin occurs in the shallow waters and widely distributed throughout the tropics and subtropics of the western Atlantic, from Florida, through the Caribbean to Brazil and Panama (Watts et al., 2001). *Lytechinus variegatus* is usually inhabits on seagrass beds and hard bottoms covered with macroalgae. It is a fast-growing urchin but the longevity is limited. Within the

age of 3 years, this species can reach to a maximum size of 92 mm TD. Average longevity ranged from 1 to 2 years. When the urchin attains a size of 40 mm TD, it gets sexual maturity within a year after metamorphosis. No reasonability of spawning was observed in this species.

2.10. Rock sea urchin (*Paracentrotus lividus*)

Paracentrotus lividus is a species of sea urchin belongs to the family Parechinidae and commonly known as rock or purple sea urchin. It occurs in the Mediterranean Sea and eastern Atlantic Ocean, extending from western Scotland and Ireland to the Azores, Canary Islands and Morocco, and most common in the western Mediterranean, the coasts of Portugal and the Bay of Biscay, where the water temperature in winter months varies within 10 to 15°C. This species usually inhabits in the shallow sub-littoral area to a maximum depth of 20 m. *Paracentrotus lividus* is intensely related to the seagrass meadows and mainly existed on encrusted rocky substratum where it makes permanent burrows to live in. It experiences with moderate growth rates and the individuals having 40 mm TD are usually 4-5 years old, and the adults with a size of 70+ mm TD are older than 12 years. The largest size of 15 mm TD is reported by Boudouresque and Verlaque (2001). The species gets sexual maturity at the size range between 13 and 20 mm TD and the spawning usually occurs during spring to the early summer months.

2.11. Collector sea urchin (*Tripneustes gratilla*)

Tripneustes gratilla is commonly recognized as collector, cake or Parson's hat sea urchin and has a circumtropical distribution, encompassing to the subtropics. This species is usually occurs in the Indo-Pacific, Hawaii, the Red Sea and Bahamas, and is widely distributed from Red sea westward to Hawaii and Clarion Island, eastward to Paumotu, as far south as Port Jackson, and at Shark's Bay on the

west coast of Australia. It is regarded as a shallow-water sea urchin and usually inhabits on a diversity of substrates and occurs in the depth range between 2 and 30 meters (Lawrence, 2007). *Tripneustes gratilla* grazes continuously during day and night and its diet comprises of algae, periphyton and seagrass. It has higher growth rate with lower longevity. Within the age of 4 to 5 years, the urchin attains a maximum size of 160 mm TD. However, it can grow to 75 mm TD in the first year of age. The collector sea urchin has an annual reproductive cycle mediated by seawater temperature, length of day and feeding activity. Spawning mainly occurs during mid to later winter months, when water temperatures and day lengths become the lowest and each clutch contains approximately 2 million eggs. Similar to the other regular sea urchins, the fertilized eggs develop into pluteus larvae, which then stay in the water column for almost 30 days. They then settle on the sea floor, undergo metamorphic induction and then become the tiny young juveniles. This species attains the sexual maturity at around 2-5 years of age to become a complete reproducing adult.

2.12. Purple sea urchin (*Heliocidaris erythrogramma*)

Heliocidaris erythrogramma or the purple sea urchin is usually distributed in the shallower coastal communities, extending from intertidal to a maximum depth of 35 m in the southern Australia. In the coastal waters of Tasmania, this species is commonly occurs in kelp communities and barrens, where it feeds by grazing and capturing drift weeds. It can also be occurred in high densities in association with sea grass beds (Keesing, 2001). Growth rates of this sea urchin usually vary depending on food availability and nutrition, but are mostly moderate. Individuals of *H. erythrogramma* attain a size of 40 mm TD within one year as well as a harvestable size (60 mm TD) at 3 to 5 years. It has been reported that individuals having maximum size of 122 mm TD

can live for more than 10 years (Sanderson, 1995). It attains sexual maturity at TD sizes of 40–50 mm within 5–6 years of age (Sanderson *et al.*, 1996). The best roe production was found to be 10-14% during August–December and spawning usually occurs between summer and autumn (Sanderson, 1994).

2.13. Shore sea urchin (*Psammechinus miliaris*)

Psammechinus miliaris is a species of sea urchin under the family Parechinidae and sometimes known as shore of green sea urchin. It shows restricted distributions in the southern and eastern waters of the North Sea, and the eastern Atlantic Ocean from Scandinavia south to Morocco, where it occurs from the low tide mark down to a maximum depth of 100 m. Its abundance is strongly associated with the presence of *Laminaria* kelp. This sea urchin is often found on or under *Saccharina lastissima*, a large brown seaweed with which it shares its range of distributions and also occurs in a variety of other habitats including under boulders and rocks, among seaweeds, on rough ground and on the rhizomes of *Zostera marina* in seagrass meadows. It is an omnivore and mainly feeds of macroalgae, diatoms, hydroids, worms, small crustaceans, mollusks and detritus. Longevity is relatively short and the growth rates are observed to be moderate. It has been reported that individuals can reach to a maximum size (45 mm TD) with an age between 3 and 4 years. *Psammechinus miliaris* gets sexual maturity in the first year at 6-7 mm TD and usually spawns in the months of spring and early summer.

2.14. White sea urchin (*Salmacis sphaeroides*)

The short-spined white sea urchin (*S. sphaeroides*) belonging to the family Temnopleuridae, is considered as one of the rare species under the group of regular Echinoids. It usually occurs in the tropical Indo-West Pacific Ocean, extending from China to Solomon Islands and Australia

including Singapore and Malaysia (Tan and Ng, 1988; Schoppe, 2000; Miskelly, 2002; Rahman *et al.*, 2012b, 2013b). This species has almost white cloudy test of 55-80 mm TD with plentiful small white spines, the size of which are between 10 and 15 mm. Some individuals have white spines with marron bands, some with all maroon species, while the others with maroon and green bands. *Salmacis sphaeroides* can be found in the depth, ranging from 0 to 90 m seawards, and also be occurred in shallow intertidal zone, particularly among seagrass meadows, coral reef substrates and in muddy sublittoral areas or washed ashore Schoppe, 2000). This species feeds different types of seaweeds, bryzoans and detritus. Spawning is found to be around the year. Following fertilization in the water column, the embryo develops into a blastula in about 9 hours. A series of larval stages follows, in which the larvae acquire more and more arms, and develops tube feet and spines within the larval body. Up to this point, the process takes about 35 days. Competent larvae swim near the surface of the substrate to determine a suitable site for settlement. After attachment, larval structures are either discarded or re-sorbed, and adult features continue to develop in the juvenile (Rahman *et al.*, 2012b).

2.15. Rock boring sea urchin (*Echinometra* spp.)

A number of recently diverged species of rock boring sea urchins belonging to the genus *Echinometra*, are widely distributed throughout the World's marine ecosystems. They occur commonly within and around coral reefs from central Japan in the north to southwest Australia in the south, from Clarion Island off Mexico in the east, and to the Gulf of Suez in the west (Rahman *et al.*, 2000; 2005). Various species of *Echinometra* exhibits circumtropical distribution and usually occur in shallow intertidal habitats, however a few has been recorded at a maximum depth of 20. These species are usually

small-bodied Echinoids, having the maximum size of 85 mm TD and can live for 8 to 10 years. They inhabit burrows and crevices and thereby defend themselves from strong wave action and predators. *Echinometra* spp. are active herbivorous in nature and without the presence of predators, they can occur in densities that exceed the primary production potential (McClanahan and Muthiga, 2001). Breeding takes place in any time throughout the year but they usually spawn during summer and autumn in warmer waters. Similar to the other regular Echinoids, *Echinometra* spp. release their eggs and sperms in the water column, where fertilization occurs externally and the planktonic echinopluteus larvae are produced through the embryonic and early larval stages. The time when the competent larvae gets suitable substratum, they first settle on the seabed then undergo metamorphosis to produce juvenile urchins.

2.16. Long-spined black sea urchin (*Diadema setosum*)

The tropical sea urchin, *Diadema setosum* commonly referred to as long-spined black sea urchin, is a member of regular Echinoids under the family Diadematidae. It is broadly distributed throughout the Indo-Pacific region, from Australia and Africa to Japan and Red Sea, extending to the Gulf of Aqaba, Gulf of Suez and Arabian/Persian Gulf (Lessios *et al.*, 2001). This species has characteristics long black spines and five white spots on the aboral side. The distinctive orange ring around its anal cone completes the special visual features of this species. It is usually an omnivorous scavenger and detritus eater and scrapes films of hard substrates. They are generally found in coral reefs and shallow rocky habitats at depths from 1 to 6 m. This species has a wide range of diets, which includes microalgae, seaweeds, coral polyps and encrusting animals (Grignard *et al.*, 1996). Gametogenesis begins in April–May, when the seawater temperature rises above 25°C in the Gulf of Suez

and spawning take place between June and September (Pearse, 1970). It has also been known to spawn both seasonally and thought out the year depending upon the locations and sites of the spawning adults. Temperature levels higher than 25°C have been observed to be a potential spawning cue (Pease, 1974). Throughout the year, the equatorial populations are found to be spawn without following any particular times. This is eventually true for the Malaysian and Philippine populations of *D. setosum* (Tuason and Gomez, 1979). In the Persian Gulf, spawning usually occurs between the months of April and May (Alsaffar and Khalid, 2000). Some other cues, such as the moon phases have been found to influence the spawning of *D. setosum*. This urchin has also been observed to trigger spawning activities in concordance with the entrance of a full moon (Lessios, 1981).

3. Culture, management and stock enhancement

3.1. Aquaculture

Mostly, the edible sea urchins are regular Echinoids (Lawrence, 2007), experiencing separate male and female sexes and are generally broadcast spawners. At the onset of the breeding season, the sexually matured adults release their gametes in the water column where fertilization takes place. The pluteus larvae form through the embryonic and early larval development of the fertilized eggs, which after a period of planktonic development, feed on unicellular diatom, settle to a suitable substratum and undergo metamorphosis to produce small juvenile urchins. At 26–28°C, almost 1 month is required to complete the larval life cycle, comprising the feeding or 4-armed stage, the 6 to 8-armed stages and finally competent stage for settlement (Figure 2). The newly born metamorphosed juveniles grow on macroalgae until attain the marketable size (40–50 mm) within the age ranged from 1 to 3 years, depending upon the species (Kelly, 2005).

Sea urchin aquaculture has successfully been accomplished on a large scale in Japan for many decades. In order to enhance the natural stocks, millions of juvenile urchins are being produced in hatcheries, for releasing to the managed areas of seafloor on the intertidal seashore areas. The nationally co-ordinated reseeding program has been developed to the extent that over 66 million juveniles were released on the reefs within which, over 80% were *S. intermedius* (Agatsuma et al., 2004). The contribution of released sea urchin juveniles to the overall catch has been estimated to be between 62 and 80%. There have also been much small-scale reseeding programs functioning in South Korea and Luzon Islands in the Philippines (Andrew et al., 2002). The farm entrepreneurs and researchers around the southern Ireland have been developing techniques for commercial sea urchin (*P. lividus*) cultivation for more than 20 years (Leighton, 1995), and comparatively newly in France (Grosjean et al., 1998). Culture of 3 commercially important sea urchins (*P. miliaris*, *E. esculentus* and *P. lividus*) has been conducted in Scotland since 1995 and there are also well-established research teams in the east coast of North America including Florida, Alabama, Maine, New Hampshire, New Brunswick and Newfoundland – working on *S. droebeckiensis* and *L. variegatus*; On the west coast of North America, including California and British Columbia (*S. droebeckiensis*, *S. franciscanus*, *S. purpuratus*); in Chile (*L. albus*); in New Zealand (*Evechinus chloroticus*), Norway (*S. droebeckiensis*) and in Israel (*P. lividus*) (Kelly, 2005).

Sea urchin brood stocks are regularly collected from the wild stocks when they reach proper sexual maturity. Matured gametes are obtained by injecting 0.5 M KCl into the coelomic cavity of both female and male urchins. Sperms in its most concentrated form are pipetted off the genital pores, while eggs are collected by inverting the female urchins over a glass beaker filled with filtered sea

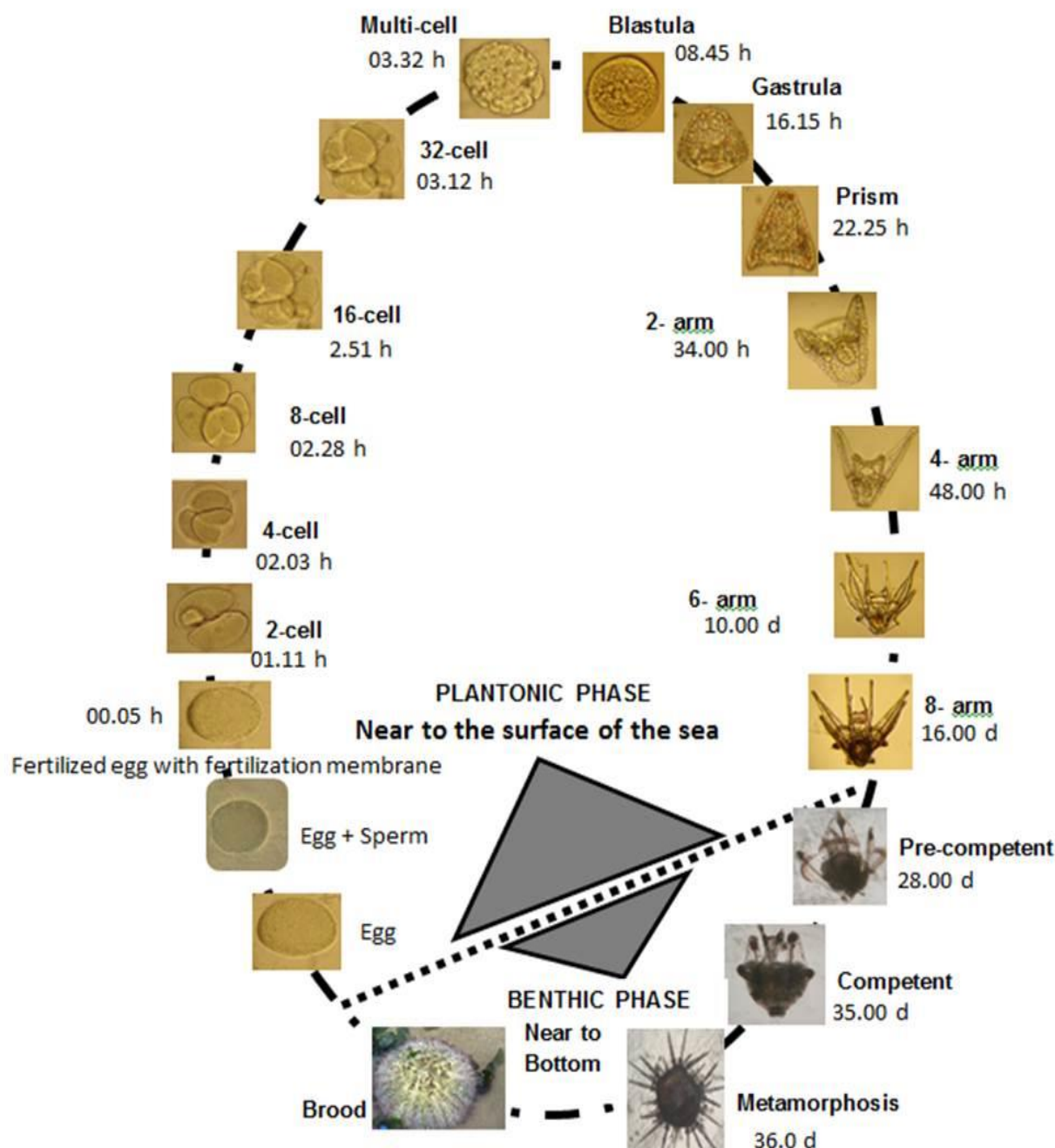


Figure 2: Breeding, development and complete life-cycle of ball-like white sea urchin (*Salmacis sphaeroides*) (Rahman et al., 2012b).

water (FSW) (Rahman et al., 2000, 2001, 2004, 2005, 2012b). At limited sperm concentrations, fertilization is usually done by mixing a few drops of diluted sperm (10^{-4} dry sperm dilutions) with egg suspensions in a petri dish and the resulting embryos are reared. Hatching of fertilized eggs usually takes 10-15 hours after insemination, to develop into a ciliated blastula. When the swimming larvae achieve feeding stage (4-armed pluteus),

they are reared in glass bottles on a rolling roller keeping a larval density of 1-2 individual/ml of medium. The unicellular cultured diatoms (*Chaetoceros calcitrans*, *Isochrysis galbana*) are commonly used as supplementary larval food at the concentrations of 5,000, 10,000 and 15,000 cells per ml of medium daily at 4-, 6- and 8-armed pluteus stages, respectively until attaining metamorphic competence within around 30-35 days post-fertilization

(Rahman et al., 2016) (Figure 3). In Japan, partial water exchange system (Sakai et al., 2004) and continuous flow-through system (Hagen, 1996) are used for the large scale cultivation. The most costly aspect of captive larval culture is the need for the concurring production of the above microalgae as live food for larvae. Nevertheless, larvae of the variegated sea urchin (*L. variegatus*) have just been confirmed to be appropriate for culturing with artificial diets (Gorge et al., 2004).

Settlement and metamorphic induction are considered as the most crucial stages for the development and culture of sea urchin larvae. The higher survival rate is always dependent on the larvae to become competent to metamorphose and then responding to the exact settlement cues. In small-scale culture, induction for metamorphosis of competent larvae has recently been performed on coralline red algal extracts + *Chaetoceros calcitran*

diatom (50:50) in petri dishes containing FSW (Rahman et al., 2012b). Within 1 day after the settlement induction, majority of the competent larvae are found to metamorphose into young juvenile (Fig. 4A). They are then reared on the encrusting coralline algal rocks in the aerated glass/plastic aquaria for at least three months by which they attain appropriate juvenile (hereafter referred to as sea urchin seed) sizes (Fig. 4D) for stocking in grow-out aquaculture system. In the countries like Japan, South Korea, Ireland, Norway, Scotland, and in British Columbia, Canada, sea urchin juveniles have been produced on a commercial or semi-commercial scale by some well-developed hatcheries and nurseries. Almost all the culturists use natural biofilm or a specially seeded diatom substratum made from species locally isolated and then grown on a PVC wave plate. However, one of the most challenging areas of

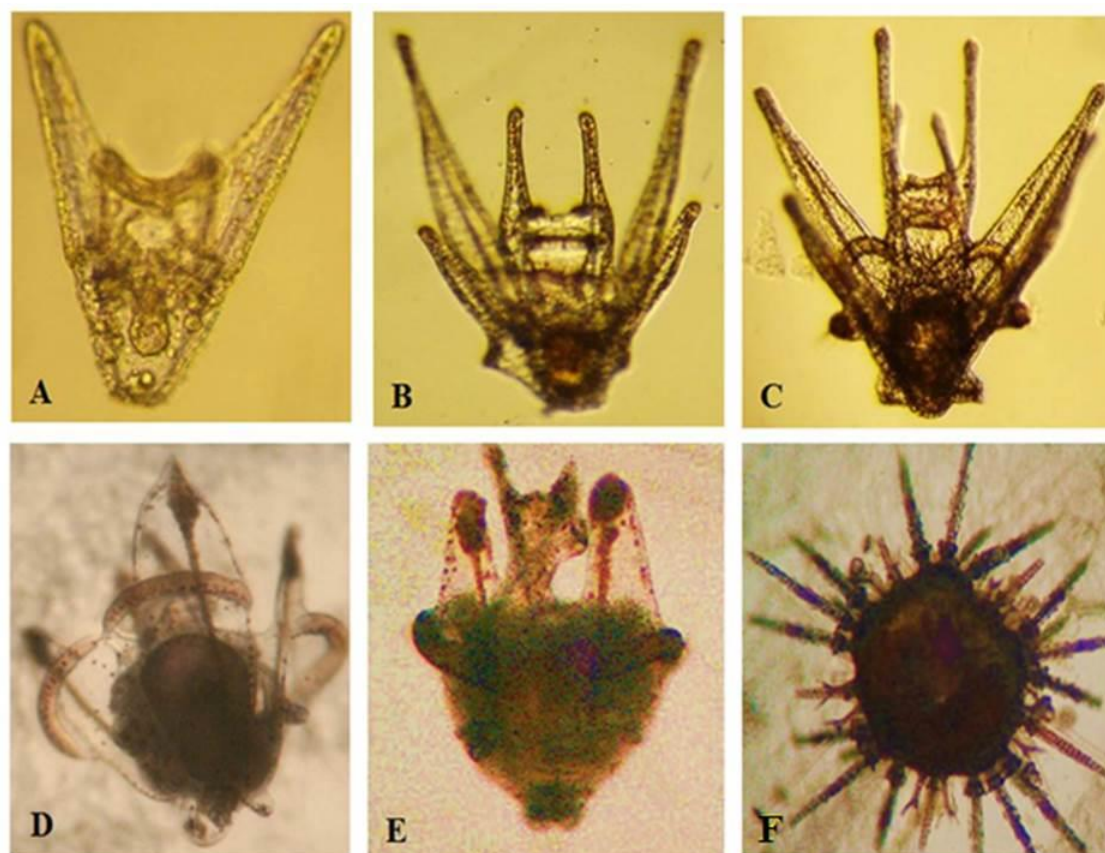


Figure 3: Developmental stages for larvae of short-spined white sea urchin, *Salmacis sphaeroides*: A) 4-arm pluteus, B) 6-arm pluteus, C) 8-arm pluteus, D) Pre-competent larva, E) Competent larva with complete rudiment growth, and F) Just newly metamorphosed juvenile with adult spines and tubefeet (Rahman et al., 2012b).

research is needed to optimize diets for the early juveniles and/or replacement of diatom biofilms. The differences in size and succeeding variation in growth rates of post-larvae remain a bottleneck in the supply of hatchery-reared juveniles. These juveniles are robust enough to survive, transfer to sea cages or other grow-out systems from a small size of 5 mm TD (Kelly, 2002; Sakai *et al.*, 2004). At this instant, they are weaned into other diets, soft macroalgae or artificial diets, depending on the grow-out culture system.

In the small-scale indoor aquaria-rearing system, 1-day-old juveniles are reared in small aquaria (25 x 20 x 20 cm) with aerated FSW and pieces of dead coral with coralline red algae are supplied as food (Figure 4) (Rahman *et al.*, 2000,

2001, 2005). Seawater is partially changed twice a month and replenished with freshly filtered sea water. The method is continued for up to three months, by which time the juveniles reach to 9.0–10 mm in TD. These 3-month-old juveniles (Figure 5A) are then transferred to glass/plastic aquaria (90 x 45 x 45 cm) provided with filtered seawater in the culture unit of the Laboratory of Marine Biotechnology, Institute of Bioscience, Universiti Putra Malaysia. Stocking density is fixed at 20 juveniles in each replicate aquarium and the urchins are fed with red alga (*Amphiroa fragilissima*), brown alga (*Sargassum polysystem*) and sea grass (*Enhalus acoroides*). Juveniles in all the treatments were fed ad libitum and seawater from each rearing aquarium was

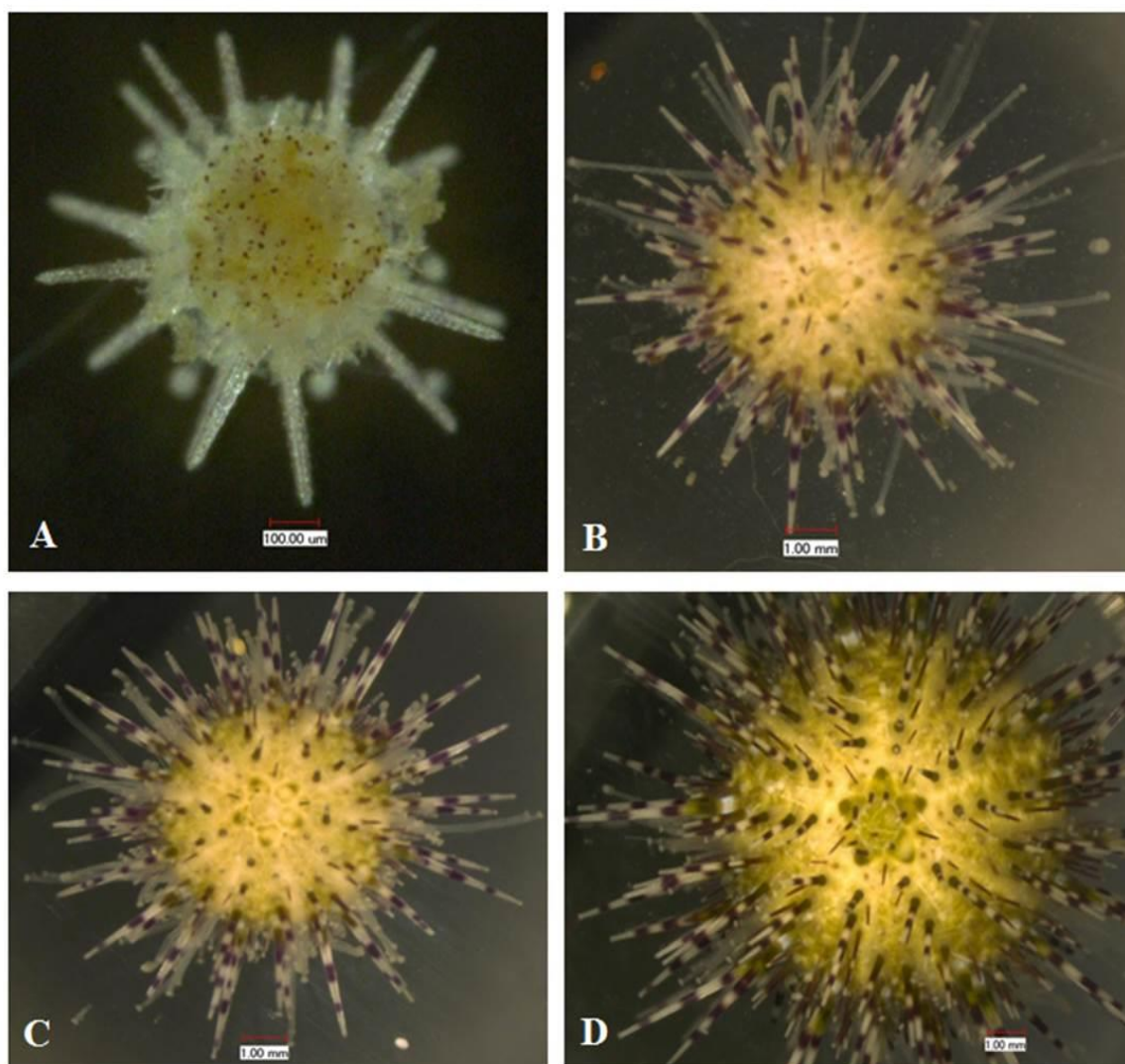


Figure 4: Juveniles of the white sea urchin, *Salmacis sphaeroides*: A) 1-day-old juvenile, B) 1-month-old juvenile, C) 2-month-old juvenile, and D) 3-month-old juvenile for grow-out culture (Rahman *et al.*, 2012b).

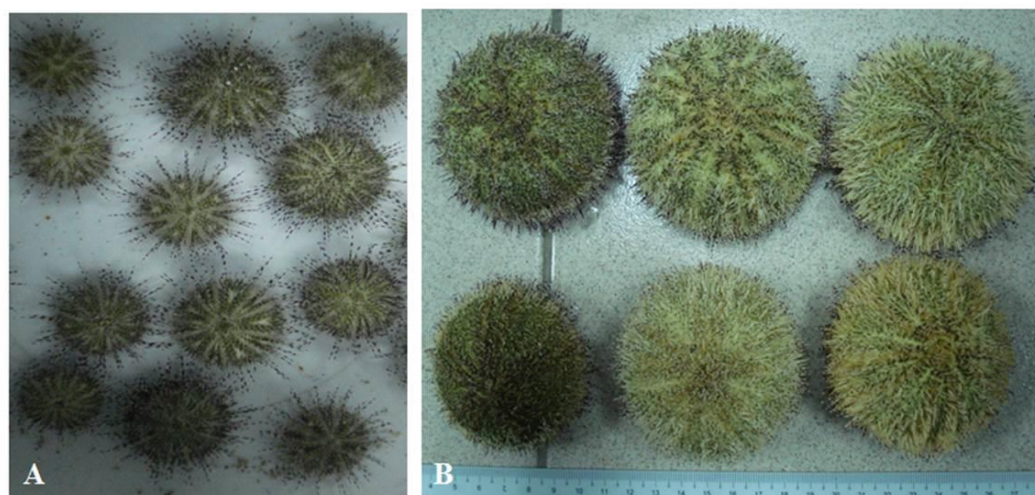


Figure 5: Stocking juveniles and cultured adults of *Salmacis sphaeroides*: A) Three-month-old juveniles for stocking in grow-out culture, B) Sexually matured adults after the culture period of two years in captive aquaria-rearing system.

Table 1: Comparison of growth and production of *S. sphaeroides* fed with different types of algae. Mean $\pm$ SE, $n = 30$

Parameters	Treatments		
	T ₁ (Red alga)	T ₂ (Brown alga)	T ₃ (Sea grass)
Initial length (cm)	10.04 $\pm$ 0.70 ^a	10.04 $\pm$ 0.70 ^a	10.04 $\pm$ 0.70 ^a
Final length (cm)	46.49 $\pm$ 1.01 ^a	43.56 $\pm$ 1.04 ^b	38.67 $\pm$ 0.35 ^c
Initial weight (g)	0.49 $\pm$ 0.11 ^a	0.49 $\pm$ 0.11 ^a	0.49 $\pm$ 0.11 ^a
Final weight (g)	51.17 $\pm$ 1.17 ^a	31.91 $\pm$ 1.42 ^b	20.80 $\pm$ 0.65 ^c
Weight gain (g)	50.67 $\pm$ 1.93 ^a	31.39 $\pm$ 1.44 ^b	20.31 $\pm$ 0.43 ^c
Length gain (cm)	36.46 $\pm$ 1.01 ^a	33.85 $\pm$ 0.66 ^b	28.63 $\pm$ 0.35 ^c
SGR (%/day)	0.73 $\pm$ 0.01 ^a	0.65 $\pm$ 0.01 ^b	0.58 $\pm$ 0.01 ^c
DGR (%/day)	7.92 $\pm$ 0.30 ^a	4.91 $\pm$ 0.22 ^b	3.17 $\pm$ 0.10 ^c
Wet gonad weight (g)	6.01 $\pm$ 0.37 ^a	3.56 $\pm$ 0.26 ^b	2.32 $\pm$ 0.10 ^c
Gonad index (%)	18.26 $\pm$ 0.51 ^a	16.44 $\pm$ 0.19 ^b	14.84 $\pm$ 0.25 ^c
Survival (%)	88.89 $\pm$ 1.93 ^a	73.33 $\pm$ 3.34 ^b	56.67 $\pm$ 5.77 ^c

Means sharing the same superscripts within the same row are not significantly different from each other ($P > 0.05$).

completely changed at every 2–3 months. After two years of culture, the urchins attain sexual maturity (Figure 5B) and those fed red alga, performed the best over the brown- and sea grass-fed urchins with regard to body growth and edible gonad production (Table 1; Rahman unpublished data). On the contrary to the Japanese culture system, where hatchery-reared juveniles are mainly released to managed seafloor (Hagen, 1996; Sakai et al., 2004; Kelly, 2005), scientists of other countries have conducted research with a wide range of grow-out culture systems for the juvenile and adult urchins, ranging

from the relocation from poor to good feeding grounds (Moylan, 1997) to the ranching of urchins caged on the seafloor (Cuthbert et al., 1995). Juveniles reared in hatcheries have been grown under suspended culture condition (Kelly, 2002, 2005) in closed recirculation systems (Grosjean et al., 1998) and in demand rock pools in southern Ireland. The sea-cage culture system of stacking baskets suspended from a ladder-like structure over which a work barge or raft can operate has been developed by Norwegian researchers (Aas, 2004). The time taken for juveniles of most species to reach market-

able size is in the range from 1 to 3 years (Kelly, 2005, Rahman *et al.*, 2014b).

3.2. Management

The sea urchin fisheries around the world have consistently shown their susceptibility to overfishing and to the problems associated with readjusting effort following a fishing-down phase. Through the late 1980s and 1990s, a number of fisheries have developed to cater for the expanding demand of the lucrative Japanese market. This has driven the development of new fisheries off Chile, both coasts of North America and Australia. These fisheries commenced on virgin stocks and as a result have needed to deal with the issues of rising expectations during fishing-down phase and adjustment of the fishery for long-term sustainability.

The current position of the various American fisheries provides the full range of outcomes in dealing with this problem. For instance, the fisheries of Canada, Alaska and Washington have had active programs to readjust effort levels and are now managed on the basis of catch limits based on sustainable harvest strategies using regular population surveys. The Californian fishery has yet to adequately adjust levels of effort and there is evidence that the fishery is now being overfished. The Chilean fishery has little capacity to readjust effort levels and it would appear that once the fish-down phase is completed there is the potential for significant overfishing. Of the fisheries reviewed here, there are numerous examples of fisheries that have collapsed to levels of one or two magnitudes below their peak production. These include the fisheries of France (Mediterranean and Atlantic), Iceland, Ireland, South Korea and Philippines. Significant parts of the Chinese fishery have also collapsed. Those fisheries that are currently being managed for long term sustainability share a number of characteristics. They are:

- limited entry (moratoriums) followed by active programs to reduce latent effort,
- resource surveys at various levels of complexity,
- the use of annual Total Allowable Catches based on resource assessment,
- zoning and area management, which may be developed to the point of rotational harvest,
- the use of minimum legal sizes.

Classical fisheries science was developed in consideration of offshore, open access and industrial fishing situations and the resulting management systems are not well adapted, or particularly robust, when applied to more complex spatial structure of small scale, inshore fishery resources (Orensanz and Jamieson, 1998). The social significance of small scale inshore fisheries is much greater, given the numbers of fishermen and other players involved, than the sometimes more productive offshore fisheries, which generally involve larger enterprises, higher capital investment and limited numbers of fishermen. As a result, much of the challenge in ensuring the sustainability of shellfish fisheries lies in developing and applying appropriate utilization, assessment and management models. According to Orensanz and Jamieson (1998), the management measures that explicitly acknowledge spatial structure of fishery resources, and are therefore the most suitable for these sorts of fisheries, include (but not limited to):

- i) territorial property and use rights including lease, traditional tenure systems etc.;
- ii) harvest rotation coupled with pulse fishing and/or thinning;
- iii) reproductive refugia and Marine Protected Areas;
- iv) experimental management with spatial control, contrasting treatments and replication;
- v) localized enhancement including habitat manipulation, seeding and predator control.

Systems that accelerate growth to market size sea urchins while producing a uniform size class would give an economic advantage. One probable way to obtain sustainable and environmentally friendly systems for urchin culture is to further examine their potential in integrated systems. They have already been shown to thrive in polyculture with Atlantic salmon (Kelly *et al.*, 1998) and to have a role in land-based integrated systems (Shpigel *et al.*, 2004). Nevertheless, many species are true omnivores, so the potential for their integration into systems where natural prey items, for instance, mussels and clams, are already produced should be explored.

3.3. Stock enhancement

Decline in world production and overfishing have prompted increasing in enhancement as a means of maintaining production. It is most developed in Japan where the 1974 Coastal Fishing Ground Improvement and Development Law provide the basis for stock enhancement (Agatsuma *et al.*, 2004). The goal of this program is to “develop and improve coastal fishing grounds systematically by the construction of artificial reefs and the release of seedlings”. Enhancement can comprise a number of different activities including direct stock enhancement through seeding of hatchery-raised juveniles, habitat improvement or restoration, creation of artificial reefs, predator control, thinning and/or roe enhancement through supplemental feeding to increase the product recoveries etc. Re-seeding has been especially applied in Japan since late 1980's. The numbers have been fairly stable since 1997 with about 70-85 million juveniles reared to about 5-10 mm TD and released each year primarily in the areas with the largest historical harvest. *Strongylocentrotus intermedius* accounts for about 85% of the urchins released by the Japanese in Hokkaido (Andrew *et al.*, 2002). Predator removal is required as excess predation by sea stars etc. has been implicated in the few cases where the re-

seeding did not have any benefit on the subsequent urchin production and crabs and sea stars are removed from the grounds using baited traps prior to the release of the urchins to control mortality in the immediate period after release. Government has taken considerable involvement in the management of coastal fisheries, particularly in the provision of subsidies for enhancement and infrastructure development as well as management coordination. A couple of studies have been looked at the contribution of re-seeding or habitat enhancement to the actual abundance of urchins in harvest areas in a sort of round-about way at localized sites around Hokkaido and estimated that re-seeded urchins comprised 62%, 66% and 80% of the total catch in 1994, 1995 and 1996, respectively (Agatsuma, 2004; Rahman *et al.*, 2014b).

Translocation of the urchins is used for a number of related reasons in Japan. In areas where kelp forest development is held back by excessive urchin densities, urchins are sometimes removed and replaced with adult kelps to permit rapid development of complex kelp forests (Agatsuma, 2004). The urchins may then be placed into intensive sea ranching pens where they are fed *ad libitum* and prepared for harvest some months down the road. Experiments have shown that Green Sea Urchins at densities up to 35 kg/m have recorded recovery increases from 6% to over 18% in 11 weeks on an artificial diet (Aas, 2004), although further finishing for about 6 weeks on a natural kelp diet is still required to get an acceptable taste profile at this point. The sea urchin *Evechinus chloroticus* is widely distributed around New Zealand but attempts to establish a commercial fishery have, like Norway, not succeeded because of the poor product quality and low recoveries. Experiments with ponding over 2 month periods have seen recoveries to increase near 20% and produced other quality improvements that promise well for the future but further research is still needed to reach an economic breakeven.

There are a number of aquaculture sites around New Zealand which are currently considered marginal for mussel farming, which would be suitable for urchin culture (Barker and Fell, 2004). Multi-disciplinary approaches are therefore needed for stock enhancement and both scientific and user group advisors should be involved (Masuda and Tsukamoto, 1998). One method of enhancement that apparently works very well with Green Sea Urchins simply requires the presence of a salmon net pen. The urchins can apparently settle out quite abundantly on such structures and grow quite nicely by feeding on the fouling organisms on the mesh. This has provided some opportunities for Canadian fishermen in British Columbia (BC) for some easy harvests.

The current market system used for the urchin trade developed in tandem with the wild fishery but this will no doubt change dramatically once cultured product is available in substantial quantities. Cultured production is more tightly controlled than from the wild fishery so that, as with the cultured salmon, the consistent availability of an invariably high quality product throughout the year will have a tremendous impact on the urchin markets throughout the world. Traditional harvesters of sea urchins do not generally know much about the potential of aquaculture (Robinson, 2004) and will likely tend towards obstructing its development as opposed to recognizing the available advantages and applying them to their own benefit. This will be unfortunate because if the wild and cultured urchin fisheries could be more closely integrated, both would stand to benefit. For example, the gonad size and quality are quite easy to manipulate and the economic yield of the roe can be dramatically and fairly easily increased. This knowledge is probably directly applicable to the wild fishery and could contribute to an increase in quality, value and profitability. Already, fisheries and aquaculture are blurring together with respect to product (gonad) enhancement and re-seeding of juveniles is coming to

the fore in a number of countries (Robinson, 2004).

4. Bioactive compounds and human health benefits

Alike many other marine invertebrates, sea urchins have been considered as a source of biologically active compounds with biomedical applications (Kelly, 2005, Rahman *et al.*, 2014b). However, the full potential of echinoids as a source of biologically active products is largely unexplored (Bragadeeswaran *et al.*, 2013). The marine environment is an exceptional reservoir of natural bioactive compounds, many of which exhibit structural and chemical features not detected in terrestrial derived natural products. The richness of diversity offers a great opportunity for the discovery of new bioactive compounds. Modern technologies have opened huge extents of research for the extraction of bioactive compounds from seas and oceans to treat the fatal diseases. The number of natural products isolated and purified from marine organisms increases rapidly and currently surpass with hundreds of new compounds being discovered every year (Proksch and Muller, 2006). The isolated secondary metabolites have numerous functions; it is likely that some of them may be pharmacologically active compounds for humans and useful as medicines (Briskin, 2000). A number of such compounds have been isolated from echinoderms (Carballeria *et al.*, 1996). There has also been much valuable information available for new antibiotics and give new insights into bioactive compounds in sea urchins. Sea urchins shells are containing various polyhydroxylated naphthoquinone pigments, spinochromes (Anderson *et al.*, 1969) as well as their analogous compound echinochrome A, of which was showed bactericidal effect as reported by Service *et al.* (1984). The phenolic hydroxyl groups in these molecules also suggested that they could participate in particular antioxidant activity as was observed in other well-known an-

tioxidant polyphenols such as tea catechins. Similar to the structured compounds are also found in the shells of sea urchins and thus suggesting that they as well as echinochrome A would act as antioxidant substances, similar to other polyphenolic antioxidants in edible plants (Chantaro *et al.*, 2008). While, squaric acid ester-based methodology was used in a new synthesis of chinochrome A was used in a new synthesis of echinochrome A, a polyhydroxylated naphthoquinone pigment, commonly isolated from sea urchin spines (Pena-Cabrera *et al.*, 2002). Gonads of sea urchins contain polyhydroxylated polyhydroxylated naphthoquinone pigments, echinochrome A, which highly potential in antioxidant activity (Lebedev *et al.*, 2001). In our recent study, we found that the ovary extracts of long-spined black sea urchin (*D. setosum*) has profound and thereby inhibit the growth of pathogenic microorganisms (Marimuthu *et al.*, 2015).

Sea urchin gonads are also very rich in valuable bioactive compounds, such as polyunsaturated fatty acids (PUFAs) and β -carotene (Dincer and Cakli, 2007). PUFAs, especially eicosapentanoic acid (EPA, C20:5 (n-3)) and docosahexaenoic acid (DHA C22:6 (n-3)), have significant preventive effects on arrhythmia, cardiovascular diseases and cancer (Pulz, 2004). β -carotene and some xanthophylls have strong pro-vitamin A activity and can be used to prevent tumor development and light sensitivity (Britton *et al.*, 2004). The composition of these valuable components, however, varies greatly among different urchin species and is influenced by their natural diets as well as physiological processes i.e., reproductive stages (Fernandez, 1998; Lawrence, 2007). On the other hand, the high levels of AA and EPA recently detected in *D. setosum* and *S. sphaeroides* greatly supported the development of aquaculture of sea urchins (Chen *et al.*, 2010), since PUFAs are important for human nutrition (Lawrence, 2007).

Based on the nutritional facts a 100 g of sea urchin gonad, which is equal to 3.5 ounces, contain 172 calories and very little fat. In fact, the fat that a serving sea urchin does contain is almost all unsaturated fatty acids. For instance, there is 1.75 g of polyunsaturated fat content in a serving sea urchin. Consumption of polyunsaturated fats instead of saturated fats, such as those found in a burger, can help in reducing the overall cholesterol level. Sea urchins also contain omega-3 fatty acids, which can help in lowering blood pressure and reducing the risk of an abnormal heart beat followed by heart attack (Rahim and Nurhasan, 2012). In addition, they serve as frequent model organism for developmental and immunological studies.

5. Bioassays for coastal water quality using sea urchin embryo-larva and adults

Coastal ecosystems are now matter to the impact of numerous human activities that lead to the input of a range of pollutants of agricultural, urban, or industrial origin. Sea urchins have been extensively used as bioindicators of marine pollution over the last several decades (Kobayashi, 1971; Phillips, 1990; Flam-mang *et al.*, 1997). The two key life stages of the sea urchin most commonly studies and used in testing are the embryo-larval and adult stages. Specifically, the early life stages of some different species of sea urchins have been demonstrated to be sensitive to metals (Kobayashi, 1973, 1980; Kobayashi and Fujinaga, 1976; Phillips *et al.*, 2003).

Sea urchins are also useful indicator species for environmental contaminations due to the fact that their sperm, embryos and larvae are very sensitive to toxins in the water (Nacci *et al.*, 1986; Paganano *et al.*, 1986; Dinnel *et al.*, 1989; Ghiradini *et al.*, 2003; Ghiradini *et al.*, 2005). They are also considered as an excellent research species because spawning and gamete collection is reasonably simple, published literatures on echinoid em-

bryological development is plentiful, the larvae develop quickly, animals are available throughout the year and are easily maintained under laboratory conditions (Hinegardner, 1969; Dinnel *et al.*, 1989; Ghirardini *et al.*, 2005). The sea urchin, *S. sphaeroides* and *D. setosum* are readily available in the Indo-Pacific including Malaysia and recently have documented embryonic and larval development stages (Rahman *et al.*, 2012b; Rahman *et al.*, 2015). Sea urchin embryo-larva development test is a standard chronic toxicity bioassay advocated to be a cost-effective and useful method for use in screening the toxicity of specific pollutants, mixtures of these and natural matrices, and has regularly been used to assess the toxicity of water sediments (Beiras *et al.*, 2003; Cesar *et al.*, 2009). This test consists of the study of teratogenic effects in early embryo to larval stages. The standardized or classical criterion for evaluating toxicity by means of this test involves distinguishing between normal larvae, i.e., pyramid-shaped larvae with skeletal rods that are half the length towards the long axis of the larvae, a differentiated gut and incipient postoral arms, and deformed larvae, i.e., larvae that display blocked or delayed embryonic development, undifferentiated or abnormal gut and absent or abnormal skeleton (USEPA, 1994; Warmau, *et al.*, 1996). However, observation of only skeletal anomalies may be more rapid, sensitive and ecologically relevant than use of the classical criterion (without considering skeletal abnormalities), which, moreover may be affected by the determining role of food availability in the larval form, rate of growth of body parts and timing of development (Strathmann *et al.*, 1992).

The accumulation of pollutants in adult sea urchins has been used to monitor contaminations of many coral reef habitats (Phillips, 1990; Flammang *et al.*, 1997). Several studies have demonstrated metal accumulation in sea urchins adequately reflects abundance and availability in contaminated waters (Augier *et al.*,

1989; Ablanado *et al.*, 1990; Flammang *et al.*, 1997). The sea urchins, *D. setosum* and *P. lividus* have been used as bioindicators for assessing heavy metal contaminations in coral reef ecosystems of the Indo-West Pacific Ocean and the north-western Mediterranean Sea, respectively (Warnau *et al.*, 1995; Flammang *et al.*, 1997). Both the embryo-larva and adult *D. antillarum* were also found to be highly sensitive bioindicators for metal pollution in marine environments on the Caribbean and should be considered when determining ecological risks in coral reef environments (Bielmyer *et al.*, 2005).

6. Livelihood development and income generation

Alike other commercially important marine invertebrates, Sea urchins offer important benefits to human beings due to their use in scientific research and education and also for food. In the economic point of view, sea urchin gonad either in the form of fresh or processed food, is considered as one of the most expensive and luxury seafood in the world (Richard, 2004). In Japan, for example, sea urchin (known as “uni”) and its processed roe can retail for as much as AU\$ 450 per kg. In addition, scientists and researchers can learn much about animal reproduction, fertilization, development and evolution by studying sea urchins, sea stars and other echinoderms as model species (Parvez *et al.*, 2016b).

The Bajau, Suluk, Kokos and Ubian tribes of Sabah (Eastern Malaysia) harvest the sea urchins, particularly their eggs, to be sold and eaten as a delicacy. This delicacy is usually prepared for special events such as Lepa-Lepa Festival, wedding ceremony and other cultural events and is being treated as valuable fishery resources in Malaysia (Rahim and Nurhasan, 2011). The sea urchins having long spines are known as “tayum” in Sabah, while the shorter spined species are called “tehe-tehe”. Sea urchins are usually sold in wet markets at different prices de-

pending on their type and location. Tatum eggs are usually eaten raw and the selling price is from RM 2 to RM 5 per pack. Meanwhile, “tehe-tehe” is sold at RM 1 to RM 2 per plate i.e., containing three urchins with their skin intact to be cooked into oku-oku, a traditional Bajau delicacy. In comparison, price of sea urchin eggs is RM 36 to RM 60 for every 80 g in America, while in Japan, an urchin can cost as much as RM 18 (Parvez *et al.*, 2016b). Aesthetically, the diverse forms of the sea urchins, and their beautiful coloring, are often providing not only a source of joy and recreation but also increase the additional revenue to humans observing them. Thus, sea urchins play a significant role in livelihood development and income generation to the local coastal communities.

7. Concluding remarks

This paper has been presented as a background document and review of the World’s sea urchin fisheries. To summarize the views, reports and publications of other scientists/researchers, it is apparent that sea urchin fisheries have a poor record of sustainability, as evidenced by the declines recorded in Japan, Maine, California and South Korea among others, as well as by the ad hoc and/or ineffective management in many sea urchin fisheries. Very few stocks have been formally assessed, meaning it is near impossible to qualify declines as the fish-down of accumulated biomass, which does not arrest the productivity of the stock, or as a case of over-fishing in which case its productivity may be forced into permanent decline. Small-scale management is mentioned time and again as offering the most promise for ensuring long term sustainability. The strong and consistent spatial structure inherent in sea urchin stocks combined with excessive effort from mobile fleets and inappropriately large scale, and therefore ineffective management all contribute to declining production in many of the world’s sea urchin fisheries.

This is mainly the case for the world’s largest sea urchins fishery in Chile, where the risks of collapse cannot be discounted. Given that this fishery alone contributes upwards of 55% of the global harvest, a significant decline in Chile’s fishery would likely lead to structural realignment in the market and higher prices for mid-range products until aquaculture production ramped up. There is also general agreement that some form of exclusive access as a prerequisite condition to promote meaningful enhancement and intelligent harvesting to maximize roe value will provide the best hedge against uncertainties in fisheries productivity and market stability. In the short term it is likely that global production of sea urchin roe from wild fisheries will decline, with the major production being provided by those fisheries that have supported active management strategies to readjust the effort and contain catches to levels that provide long-term sustainability. Given that demand is unlikely to decline; future production will be increasingly valuable. In order to make sea urchins fisheries viable and profitable, the following actions are suggested:

- Refinement of artificial diet formulations for juveniles and adults to maximize the growth rates and survivorship and produce gonads of the desired taste, texture, flavor and color.
- Optimization of grow-out facilities for juveniles and adults either at sea (in containers and ‘ranching’) or land-based.
- Regulations regarding fishing methods, fishing areas and protection of company investments need to be developed.
- Better surveillance of sea urchin density to guarantee a steady flow of raw materials.
- Areas need to be thinned out to get the best possible product for the market, this is also necessary for the kelp forest to grow back.
- More capital needs to be directed towards investing in technology for

processing to reduce labor costs and preserve product quality.

- Improved cooperation between fishermen and processors, when marketing and selling the sea urchins.

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Marine Pollution and Its Impacts on Living Organisms

Thavasimuthu Citarasu* and Mariavincent Michael Babu

Centre for Marine Science and technology, Manonmaniam Sundaranar University,
Rajakkamangalam- 629 502, Tamilnadu, India; michaelmsu@live.com (MMB);

*Correspondence: citarasu@gmail.com

Abstract: Marine environments are seriously affected by different pollutants created by human activities. The main entry of pollutants to marine environment is through atmosphere, water bodies, ships and other human activities. The pollutants seriously affect the marine flora, fauna and disturb the food supply chains. Several synthetic chemical residues including carbon tetrachloride, polychlorinated biphenyls, trichloroethylene and vinyl chloride are found in the marine sediments and flora and fauna. This type of marine pollution does have direct or indirect effect on human health. The biotechnological approaches such as bioremediation, probiotics, waste treatments by micro algae and seaweeds are useful to restrict and reduce the pollutants in the effluents before they reach the marine water bodies. This article highlights various aspects of marine pollution and its impacts on living organisms. Several pollution preventive measures and awareness programs are also discussed in this chapter.

Keywords: Marine biotechnology; marine ecosystem; marine pollution; microplastics; pollution awareness

1. Marine ecosystem and its resources

The ocean occupy 71 percentages of earth's surface; they are interconnected and traditionally divided into four large basins including North and South Atlantic, North and South Pacific, Arctic and Indian oceans. The average depths are 13,216, 10,932, 12,786 and 3,665 feet for Pacific, Atlantic, Indian and Arctic oceans, respectively. Marine environment is most important for life on earth, the living organisms originated in marine and they emigrated to terrestrial and freshwater bodies. Oceans are the main regulators of climate and temperature to the terrestrial ecosystem. Phytoplanktons are important for oxygen production; they yield around eighty percentages of oxygen which is used by the animals and plants for breathing in terrestrial and aquatic ecosystems (Bigg *et al.*, 2003). Marine ecosystem is the largest ecosystem with intertidal zones, coral reefs, estuaries, la-

goons, salty marshes, mangroves, deep sea and sea floor ecosystems which are important for marine (and terrestrial) living organism (Barange *et al.*, 2010). They provide goods and various services to the human society such as good climate, vital foods, medicines, bio processing and employments including fishing, process industries, aquaculture and coastal tourism etc.

2. Marine pollution

The oceans are susceptible for pollution ever by human activities by polluting with different agents and physical destruction ocean environments. The definition of Marine Pollution as “*Introduction by man, directly, or indirectly, of substances or energy to the marine environment resulting in deleterious effects such as: hazards to human health, hindrance to marine activities, impairment of the quality of seawater for various uses*

and reduction of amenities". Marine pollution is mainly classified to coastal sources by riverine inputs, deposition from atmosphere and offshore inputs etc. Coastal diffuse sources are contaminants via coastal industry, sewages and development sites (Clark, 2001). Alternative inputs are site-specific discharges, agriculture lands and forests, mainly for nutrients leakage to groundwater bodies, further they transported into the marine ecosystem by backwater bodied and finally huge quantities of contaminants deposited marine environments (GESAMP, 1993). Municipal wastes and sewage water effluents are not properly treated and discharged in to sea, these problems mainly happened in developing countries. Emissions through air craft's are main sources of atmospheric pollution to sea and the pollutants are dispersed a vast areas by the wind flow and weather changes. Marine pollution in offshore caused by discharges of pollutants from vessel based at least 10 % of the total marine pollution and they contribute by different ways (Anon, 2005). The other sources are including crude oil extraction and of mineral extraction etc.

3. Pollutant Sources

3.1. Oil pollutants

Hydrocarbons are classified into alkanes, naphthenes and aromatics. The crude oil also contains nitrogen, oxygen and vanadium compounds. The spillage of oils from the ships/ cargo, platforms of offshore oil and on-shore refineries, it may be produced serious effects to the marine environments in multiple ways. The causes of oil pollution produce alterations in physic chemical levels, also involved toxication of marine habitats and the flora and fauna seriously affected aftermath of large spills. The turbidity of oil prevents the light penetrations and lead to photosynthesis processes for phytoplanktons. Lose of waterproofing qualities faced by the larger animals, aquatic birds getting lost of waterproofing by losing

their feathers (Kachel, 2008). The crude oil is highly toxic to the marine organisms because of it contains toluene, xylene, benzene and polycyclic aromatic hydrocarbons and these chemicals bioaccumulated to planktons, fishes, shellfishes, sediments constitute a long lasting threatening to the benthic animals. Polycyclic Aromatic Hydrocarbons (PAHs) is having many pathetic effects to the living organisms including mutagenic, carcinogenic and act as a teratogens (Kachel, 2008).

3.2. Persistent toxic substances (PTS)

Persistent Toxic Substances" (PTS) also called Persistent Organic Pollutants (POPs) are noxious, long-lived and less persistent. The prolonged usage and dispersion of PTS may cause serious problems and responsible for chemical and biological degradation. Their physical characteristics are chlorinated or halogenated affected to water solubility levels and high lipid solubility, no degradability leading to fatty tissues bioaccumulationsn (El-Shahawi *et al.*, 2010). POPs constitute remarkable societal advantages, or not planned by-products of burning processes, such as dioxin. The halogenated hydrocarbons derivatives of tributyl tin (TBT), dibutyl tin and monobutyltin that are the disruptors of endocrine organs (Kachel, 2008).

3.3. Heavy metal pollution

Through riverine input, heavy metals are entering the sea and accumulate in marine sediments as well as flora and fauna. The important heavy metals are mercury (Hg), cadmium (Cd) and lead (Pb), accumulations are important for toxicity (Burger and Gochfeld, 2002). They generally share the features of PTS, because they are bioaccumulate, non-degradable and generate stringent or long standing toxic effects. The toxicity effect are vary based on the heavy metal types, for example if absorption of mercury as in very little doses, cause severe harm to brain and the central nervous system. The main sources of oceanic and atmospheric

contamination heavy metal contaminations provided by electric utilities, fuel combustion, iron manufacturing, incineration of urban refuse and fuel additives. Marine anti-fouling paints are very dangerous to marine organisms because it contains. Lead is another big poison creates the problems like neurotoxicity, mental health problems, lead is mainly released from batteries, sewage and fuel combustions. Electroplating factories, batteries and sewages are also responsible for cadmium toxicity and it affects bones by deformities and kidney dysfunction. Nickel poisoning is also a potent carcinogen that is toxic at relatively low concentration. Crude oil contains selenite with sulfur, reported as a toxic metal found out in marine environment at a huge level. The other dangerous heavy metals contaminations released through various routes including rain of pollutants from the atmospheric air, fallout from ship destruction and contaminated land runoff creating dangerous environmental problems (Kennish, 1998).

3.4. Thermal pollution

Nuclear reactors release huge amount of heat to the marine environments leading to decreased level of oxygen can disastrous effects on ecosystems and its communities. The decreased level of oxygen in marine water, degrade the quality of wildlife animals that lives underwater. The increased temperature holds less oxygen and creates suffocation to marine fauna including copepods and amphibians (Gautam *et al.*, 2016). Industries are responsible for decrease the quality marine life and destroy habitats by altering the natural environments. Natural destruction including geothermal activity and volcanoes under the marine can induce warm lava to increase the temperature of marine waters. Thunders and lightening is also responsible for tremendous level of heat into the marine that leads to raise the temperature of marine water. The high temperature discharge from industries is responsible for induc-

ing the toxin secretion from the microbes and harmful algal blooms in the marine water create other environmental problems. Environmental changes causes particular animals can change their living place to alternate place due to warmer waters and reproduction also affected by increasing temperature. Marine organisms lay of undeveloped eggs or the temperature prevents the normal development of particular eggs due to rise of temperature level in marine waters. Enzymatic activity and metabolic rate also raised and increased food consumption due to increase temperature. This is seriously affecting the stability of food chain and modifies the balance of species composition (Brett, 1970).

3.5. Nuclear radiation

In marine environments, radiations may classified in to natural and human activities, natural radiations are emission of cosmic rays, potassium-40 by earth's crust and decay products of uranium etc. Human activities including nuclear power plants and reprocessing, nuclear weapons testing and accidents, fallouts of nuclear wastes, radiation using food conservation, medical diagnosis combustion, land-based mining, phosphate production, and oil exploration etc (Shinde and Gawande, 2015). High level radioactive waste dumping is not permitted in the ocean, even low level wastes is still permitted to the marine trenches, because low level wastes containing low radioactivity than high level waste. High level nuclear waste had half-life for 24,100 years whereas the short-lived radioactive elements had the half life of 30 years. The radioactivity may absorbed by micro algae, zooplankton, and other small marine organisms and then this will be transmitted or biomagnified to the food chain, to fish, marine mammals, and humans. In human concerns, the radiations cause cancer, changes in 'DNA' that ensures cell repair (Kachel, 2008).

3.6. Excess nutrients

Emissions and discharges of agriculture sources are a significant pollution to the coastal zone particularly affects biogeochemical cycles and primary producers. Eutrophication is created by the pollutants including discharge of ammonia and methane, use of insecticides and other pesticides affecting marine flora and fauna. Excess nutrients discharges including phosphorus and compounds are responsible to eutrophication. High concentrations of nutrients, based on the physico-chemical properties of the affected marine environments, may lead to increased growth of phytoplanktons (Sarkar and Malchow, 2005). Hydrogen sulphides also induce the negative impacts, if the oxygen level decreases, hydrogen sulphides levels also increased. Hydrogen sulphides induced low resistance to the aquatic living organisms leading to die off. The dead micro algae due to hydrogen sulphide toxicity floats on the sea surface leading to interfere the penetration of sunlight. Fertilizer runoff during heavy rain the organic fertilizers run off from the agricultural field and it affects the marine environment and back water bodies.

3.7. Microbial contamination

Generally microorganisms are released from waste water, waste products and sometimes human and animal wastes into the environment seriously spoiled the marine ecosystem. The viruses do not replicate without the help of host animals. First it may infect some other host cells and multiply. Seafood contaminations are the important pollution. The effluents from fish processing units, shrimp farms, hatcheries are creating big problems. The effluents contain serious bacterial pathogens such as salmonella, pathogenic *Vibrio*, WSSV and the fungal pathogen *Fusarium*. Due to the broader host range of these pathogens it may infect very easily in the marine animals and affect the food chain. This also affects the consumer levels very easily and there is an easy to

get the diseases such cholera, typhoid etc (Yazhini *et al.*, 2015).

3.8. Noise pollution

Life in marine can be susceptible for noises from different noise pollutants including oil exploration, seismic surveys, passing ships, and naval low-frequency sonar waves. Sound is very fast in water than in the atmosphere and the noise pollution in marine are increased at ten folds during the period of 1950 to 1975. Marine mammals including dolphins, porpoises and whales are using sound as communication and sensation because sound is not or less effective for their communications. Also the particulates scattering the light and it interferes the communications in marine mammals. Also it is impossible to using smell as communications, because the chemical molecules in water diffuse more slowly than in air are not possible (Payne, 1983). Sound speed is four times higher in water than atmosphere so it is opt to communicate the marine mammals. Sonar waves is very dangerous to marine mammals it interfere the sound wave communications and causes severe injuries including lose of sense organs leading to death. Also the sounds emanated from the passing ships other instruments for marine mining activities also disturbed the dolphin and whale populations.

3.9. Ship based threatening

Ship based threatening mainly caused by operationally or accidentally and releases the pollutants to the marine ecosystem and these pollutants mainly damage the flora and fauna. The pollutants also somewhat released more in ship are on voyage than accidental ships. The pollutants released including tank residues, bunker oils, chronic discharge of sewage, garbage, exchange of ballast water, other emissions from vessel and anti-fouling paints etc. The sewage discharges causes severe problems including microbial pollution especially bacterial infections it will be seriously damage the biodiversity, fisheries and food chains etc.

The excess nutrient discharges also affect the primary production etc (Katchel, 2008). Pollutants are released by accidental pollution including contacts with external objects, due to collisions, cargo-transfer failures, sinking or loss of cargo, explosions and groundings etc. It is also very hazardous, the toxic materials spilling from cargoes which carry large quantities. The oil tankers happened in accident may spill larger quantities of crude oil to the marine environments. The oil may drift to the sea shore and seriously affected the ecosystem particularly the rocky animals including oyster and mussel beds, backwater etc. The places which affected by oil spills may suffer a long times, until the oil pollution completely degraded or solved (Katchel, 2008). The hydrocarbons from the oil spills penetrates the marine and related ecosystems sediments alter the community structure especially the phytoplanktons and worms. Ship can also harm to the marine habitats and wildlife by damaging the marine animals by physical destruction by anchoring. Anchoring may seriously affected by the coral and sponge beds. Another physical hindrance including collisions by ships, ship's propellers and ship strikes are cause the damages and death to the marine mammals including whales etc.

3.10. Harmful algal blooms (HABs)

Harmful algal bloom (HABs) causes pathetic effects to other marine animals by mechanical damage through the secretion of toxins. HABs are involved in large-scale marine mortality of marine animals and they secreted with different toxins responsible for shellfish poisonings (Nancy, 2012). Red tides caused by HABs that can affect estuarine and salt lakhs areas by depletion of oxygen, increasing the carbon dioxide and secrete toxins. Thousands of harmful algal species were identified among that one hundred species were produce toxins that will seriously affect the shellfish species by ingested the HABs by filter feeding. The excess amount algal bloom may dies off

and deposited the sediments may add dead organic matter load. Further dead organic matter may cause bad effects to the normal microbial flora and depleted the oxygen and this will affect the decomposition process. Shrimp farm effluents including algal bloom die off, large amount of dead organic load accumulate, affect filter feeding animals, shrimps and lobsters.

3.11. Ocean mining

Deep sea mining is the mineral retrieval process from the ocean floor by disturbing the marine organism from benthic regions. Ocean mining takes place about from 1,400 to 3,700 meters below the oceans' surface for extinct or active hydrothermal vents (Ahnert and Borowski, 2000). The mine deposits are drill out from the ocean deeps by hydraulic pumps or buckets that take ore to the surface. Removal of marine floors disturbs the benthic habitats. Removing parts of the seafloor disturbs the habitat of benthic organisms depending upon the places and type of mining; sometimes it may disturbances to the marine benthos permanently. Leakage, corrosion and spilling might be altering the area of mining by contamination of chemicals. Surface plumes may cause more serious effects and based on the particles size and water currents by the plumes could spread over vast areas of the floor. Sand mining is a practice mined from beaches, inland dunes and dredged from ocean beds and river beds. Sand mining also emits radiation problems and pathetic effects to the living organisms in sea shore peoples (Pitchaiah, 2017).

3.12. Plastics and polythenes

Plastic pollution is one of the serious concerns in recent years because the plastic pollutions in ocean become significant environmental issues related to governmental and nongovernmental organizations, scientists and members of the public worldwide. The main risk to marine biota posed by different activities in-

cluding marine vessels, commercial fishing, marine-industries and recreational coastal tourism. These are the major sources for generating the plastic and polythene wastes that can directly enter the marine environment. During World War II end, approximately eighty percent of the marine debris is considered as plastic wastes.

Marine vessels have been reported as a major contributor of marine litter and it is estimated that the commercial fishing fleet dumped more than 23,000 tons of plastic packaging materials during 1970s (Pruter, 1987). The waste materials including nylon netting, plastic monofilament line and discarded or waste fishing gears are neutrally buoyant can therefore drift at variable depths within the oceans. The waste plastic materials are problematic to marine animals because they cause entanglement of marine biota, known as “ghost fishing” (Lozano and Mouat, 2009).

Waste six pack rings, plastic bags and other forms of plastic waste cause dangerous effects to wildlife and fisheries. Plastic fishing nets can be lost or left by fishermen to the ocean are dangerous and create so many problems including entangle to fishes, sea turtles, sharks, dolphins, crocodiles, crabs, seabirds, and other creatures, restricting movement, causing starvation and infection etc. Plastic waste debris during bulky or tangled, it is very difficult to move, and may become permanently trapped in the digestive tracts of marine animals finally leading to blocking the food passage and causing death through infection or starvation. Moser and Lee (1992) conducted a study for plastic pollution in sea birds, they collected 1033 birds and among the birds, fifty five percentages of the birds guts containing plastic particles. Carpenter *et al.* (1972) observed different species of fishes had plastic wastes in their guts especially white plastic spherules had been ingested, indicating that they feed selectively. The same pattern of white plastic debris ingestion also observed in logger-

head sea turtles, *Caretta caretta* from Central Mediterranean sea (Gramentz, 1988). Polythenes is the another dangerous pollutants Generated from household, industrial wastes and Recreational beaches. Sea turtles mistakenly eating the polythene bags. During degradation of polythenes, it releases phenol, bisphenol, phthalate and gases it causing cancer, heart failures in humans.

3.13. Microplastics

Microplastic contamination in ocean has been a serious issue nowadays with harmful effects to marine biota. They are very tiny plastic granules derived from the breakdown of macroplastics and the plastic particles used in cosmetics and detergents. Primary microplastics in personal care and cosmetic products are a minor source of releases of microplastics to the environment. Microplastics which used in detergents, cosmetics and also in air-blasting media are entering the freshwater bodies and reached to domestic or industrial drainage systems (Derraik, 2002). Most of the microplastic granules are trapped the waste-water treatment plants unfortunately the small microplastic granules from cosmetics and detergents are passing through such filtration systems and finally reached the marine environments (Browne *et al.*, 2007). The small size nature, microplastics are considered bio-available to marine organisms throughout the food-web. Microplastics in intertidal sediments have been shown to reduce the thermal conductive properties and alter drainage. Due to the tiny size and availability of microplastics in pelagic and benthic ecosystems, they have easily ingested by the marine biota including microalgae, crustaceans, fishes and sea birds etc (Blight and Burger, 1997). Devriese *et al.* (2015) studied the microplastic contamination in the brown shrimp, *Crangon crangon* sampled Europe and the study revealed that, the shrimps are heavily contaminated with microplastics. Ingestion of microplastics may release toxins to the food chain and

lead to the bioaccumulation and biomagnifications. Additives used in microplastics such as polybrominated diphenyl ethers, phenol and bisphenol-A are inhibit the synthesis of endogenous hormones leading to affect the reproduction. Phthalates are associated with broad range of genotoxic damage including micronuclei and apoptosis in mussel haemocytes, suppressed the locomotion in invertebrates and intersex conditions in fishes (Oehlmann *et al.*, 2009). Consumption of microplastics and nano-plastics by humans through marine foods including shrimps, shellfish and small fishes may cause health problems. Ingestion of microplastics by young fishes causing deaths, stunted growth, altering behavior, sometimes killed and prevented from reaching maturity.

3.14. Ocean acidification

The oceans are act as a carbon sink, because they absorbing more carbon dioxide from atmosphere. If the carbon dioxide levels increased in the oceans the Ocean become more acidic. The acidic nature is seriously affected by the formation of calcium carbonate in corals, shellfishes including shrimps, oysters, mussels, clams and other molluscs etc shells (Caldeira and Wickett, 2003). The higher acidity in the seawater also creates more pathetic effects to the marine organisms in combinations with environmental stressors including higher ocean temperature and other pollutants etc. Marine organisms like algae and zooplanktons using carbonate ions to produce calcium carbonate shells and skeletons. Unfortunately, the acidification leads the less availability of carbonate ions in marine waters and it interfere the formation of shells. Also the ocean acidification interfere the iron, phosphorous, nitrogen and other elements absorption from marine waters by the marine organisms for their vital growth. More acidity interfere the attachment of iron to other organic compounds may hindrance to the normal marine life.

3.15. Coastal tourism

Coastal tourism creates so many harmful effects to the marine environments adding pollution by waste disposal, input of local waste structures and habitats under enormous pressure. For new tourists, over developments including construction of resorts, marinas, golf courses and airports etc are not advisable to clean environments. For the new developments peoples removed the mangrove forests and sea grass meadows. Piers and other related structures are built directly from the top of coral reefs related to the tourist developments is not advisable and spoil the marine environments. The overcrowding of tourists in the beaches may affect the nesting sites of the marine endangered turtles by destruction. Resorts from beaches may discharges their sewage effluents directly to the marine waters which is seriously affect the coral reefs and other sensitive marine habitats. Coral reefs also damaged by different tourist's activities including fishing, snorkeling, diving and careless boating etc. Boating and other human activities also disturbed the marine animals including seals, dugongs, whales, whale sharks, dolphins and marine birds. Overfishing in a particular area for seafood consumption affected local fish populations (Sunulu, 2003). Tourists also discharges the polythene bags, bottles and other plastic materials to the beach by improper disposal and it reaches to the sea create so many environmental problems. Tourism can cause pollutions including solid waste and littering, releases of sewage, oil and chemicals, air emissions, noise and even architectural activities. Tourism development of marinas and breakwaters also can changes in currents and coastlines.

3.16. Ballast water

Ships need ballast to maintain the balance successfully and safely. Discharges of ballast waters from ships to marine environment have negative im-

pacts to various ways. Huge quantities of waters are loaded by cruise ships, cargos and large tankers from one place for balancing and discharge to some other places. Ballast water have various biological materials such as seaweeds, sea grass, phytoplanktons, zooplanktons, small fishes, invertebrates, microbes including bacteria, fungi and virus etc. The biological materials are nuisance, non-native and exotic species can create extensive ecological and economic problems to the aquatic ecosystem with human health issues etc. The important preventive measures for treating the ballast water by treating with ultraviolet irradiation, filtration, heat, gas super saturation and electrical fields etc (Silva *et al.*, 2004), chemical treatments by iodides, borates, chlorates, ozone, ionization, copper ions, hydrogen peroxide, adjustment of pH, changes in salinity for eradication of organisms, ozonization and deoxygenization etc (Wright *et al.*, 2003).

3.17. Natural calamities

Hurricanes and floods can induce waste transportation from land to the marine environment. Earthquakes and tsunami can cause ground, air, and water pollution (Shaw, 2006). The pollutants including detergents, chemicals from processing plants, farm wastes and fertilizers from crops are swept downstream and deposited to marine through large river floods. Volcanic eruptions have also been creating fluorine-containing compounds deposition to sea and also harm to the marine flora and fauna. High amount of minerals are accumulated to the marine environments from demolishing of the buildings, bridges during natural disasters and small amount of hazardous materials reached to marine environments that threatening to marine living organisms. Heavy floods in rivers also washed out the sewages to the accumulated in the marine environments that contains pathogenic bacteria fungi and virus which causes serious pathological effects to the marine

living organisms and passes through the food chain.

3.18. Marine littering

Littering to marine environment is a global concern, they affect ecosystem very seriously. Several million tons of litters are discharged to the worldwide every year creating economic, environmental, health and aesthetic issues (Strieker, 1998). The important land based disposals including industrial outfalls, rivers and floodwaters, discharge from storm water drains, untreated municipal sewerage, land-fills and littering from coastal tourism. The marine based litters including shipping transport, offshore mining and extraction, fishing industry, illegal dumping at sea and discarded fishing gear etc. The vast majority of marine litter is plastic, which never truly breaks down. Another serious problem is nuclear waste disposal to the marine trenches, Mariana trench is the main site for nuclear waste disposal because of its huge depth 36, 201 feet which is situated in the western Pacific Ocean, to the east of the Mariana Islands (Sheavly and Register, 2007). Ocean is used as a dumping ground for disposing the nuclear materials for many decades after Second World War II, and then it was banned internationally. Thirteen nuclear capable countries are dumped their nuclear/radioactive waste including started from 1946 to 1993. The important nuclear waste materials are medical products, industrial products, weapons, house hold containers, reactor vessels, with and without spent or damaged nuclear fuel etc. The discarded wastes emit nuclear radiation leading to health issues to the marine living organisms.

4. Impact of pollutants to marine life

The pollutants which are affect a broad range to the environments as well as the living organism in the entire world directly or indirectly. Climate changes are spoiled by extreme weather conditions

as well as rising of sea level, sea surface warming by temperatures and ocean acidification. Corals reefs are important for preventing acidification etc, loss by anthropogenic stress activities, collection and recreational activities and coral beds may be affected. Mangrove forest is important for preventing barrier for the natural calamities like tsunami. Degradation of mangroves by over exploitation may affect the marine environments. Illegal and over exploitation of capture fisheries by trawlers may spoil the fishery resources leading to decline the particular fish species. The pollution in beaches by coastal tourism causes several environmental impacts and the oil pollution causes reduction in benthic organisms. Biodiversity is important for produce organic material, decompose organic material, capture and store energy, cycling water and nutrients and helps to regulate climate and atmospheric gases. The pollutants seriously interrupt the food chains leading to several problems. In concern with the human health point of view, the chemical poisoning in food chain creates the bioaccumulation and biomagnifications problems (Table 1). The harmful chemical contamination in seafood cause several problems to humans. The pathogenic bacteria including pathogenic vibrios, salmonella contaminations from waste water disposal from fish processing industry may cause the disease problems in the fishes/ shrimps from the sea. The consumption of infected fish/ shrimp may cause diseases to humans. The residue of

pesticides may change hormonal system, reduce fertility, weaken immune system and create cancer. The degraded derivatives like phenol, bisphenols and phthalates also creating heart diseases and cancers to humans by consuming sea foods.

5. Remedies to solve the pollutants by biotechnological approach

Bioremediation is defined as “*The act of adding materials to contaminated environments such as oil spill sites, to cause an acceleration of the natural biodegradation process*”. The microbes utilize the nutrients from the polluted water bodies and reduce or neutralize the pollutants. The extremophilic microbes including halophilic, thermophilic, acidophilic and alkaliphilic are useful to bioremediation purposes for to neutralize the pollutants, because they withstand high temperature, low and high pH and higher salinity. The polluted effluents/ water bodies, fish farm effluents, effluents from any waste sources may remediate with microbes before reaching the water bodies like river, back water may help to reduce or remove the pollutants and solve the pollution problems. Genetically modified microbes like super bug also helps degrade the oil from oil contaminated water bodies.

The water probiotic microbes like *Lactobacillus* sp and *Bifidobacterium* sp also clean the aquaculture ponds by reducing or removal pathogenic microbes

Table 1: Impact on human health by some synthetic chemicals detected in ocean

No	Chemical residues	Human health impacts
1.	Benzene	Anemia, blood disorders and chromosomal damage
2.	Carbon tetrachloride	Cancer; central nervous system, liver, kidney and lung damages
3.	Dioxin	Cancer and skin disorders
4.	Ethylene dibromide	Sterility and Cancer
5.	Polychlorinated biphenyls	Kidney, lung and liver damages
6.	Trichloroethylene	CNS, cancers, liver and kidney damage and skin problems
7.	Vinyl chloride	Cardiovascular problems, liver, kidney, and lung damages and gastrointestinal problems

and unwanted physicochemical parameters which solve the effluent contamination to the marine environments. The micro algae like *Chlorella* sp., *Ankistrodesmus* sp. are also useful to removal of excess nutrients and CO_2 in waste water systems, Solve BOD problems by releasing oxygen which will help to effluent treatment. The seaweeds like *Sargassum* sp also helpful to chelate the heavy metal contaminates in the water bodies especially the effluents. These types of treatments which restrict the polluted effluent water to the marine environments.

6. Suggestions to protecting marine environment

Several measures for protecting the marine environments from the pollutants including legislation for plastics and polythenes, set standards protocols for sewage and other effluent discharges, low level use of pesticides, coastal cleaning programmes, public awareness, restrictions of polluter pays principal and implementation of laws pertaining to prevention by strictly and coastal zone management activities before constructing new industries on the coast. Also strictly advice to the peoples for following the marine act s including National Marine sanctuaries Act of 1972, Fisheries Management and Conservation Act – 1976, Clean Water Act of 1977, Endangered Species Act, Oceans Act of 2000, Estuaries and Clean Waters Act of 2000 and Estuaries and Clean Waters Act of 2000. It is also important to conserve the marine ecosystem and marine animals we must follow the international marine legislation such as Convention on the Prevention of Marine Pollution by Dumping wastes and Other Matter. One of the important legislation, Protocol to the International Convention for the Prevention of Pollution from Ships (MARPOL) address the marine pollution problem caused by marine vessels to the marine environment in 1978 (Lentz, 1987). Education is also a very important and powerful criterion to ad-

dress the pollution problems, especially for school students and create awareness. Because school students and youngsters take their responsibility and awareness to the public peoples, families and related communities related to the marine pollutions and the future conservations etc. Coastal cleanup is the major awareness programme, the International Coastal Cleanup is one of the largest volunteer program in worldfor cleaning the coastal areas. Volunteers are clean or remove trash from the coastal areas especially beaches the entire world. Waste management is also an important strategy for restrict the entry of pollutants through effluents to the clean water bodies. Industries are advised to set up waste proper treatment plants, minimize waste for adopting suitable measures, waste recycling and reuse and recovery of waste water effluents.

7. Concluding remarks

The entry of different pollutants from atmosphere, through water bodies, by ships, from beaches and other human activities are very harmful to the marine environments and affect all marine flora and fauna. The pollutants also directly or indirectly affect the human community through food chain. Among the pollutants plastics and micro plastics are the serious concerns to the marine environments and affect lower to higher organisms and indirectly affect human's health. Restriction or reducing the pollution is in our hand, we can take care of the environments by avoiding pollution to save our environments for future. Several pollution preventive measures can be adopted including picking up and disposing various types of litters at proper place; by reducing, reusing and recycling the waste materials; bring reusable, biodegradable bags to the grocery stores; disposing properly the trash; promoting awareness among your friends and family; restrict the usage of plastic bags; awareness programs to the public especially the school children, un-

educated publics to restrict / stop the pollution will definitely help in minimizing the pollution.

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Ecology, Distribution and Diversity of Bioluminescent Bacteria in Palk Strait, Southeast Coast of India

Srinivasan Rajendran¹, Ganapathy selvam Govindarasu² and Govindasamy Chinnavenkataraman^{1,*}

¹Department of Oceanography and Coastal Area Studies, School of Marine Sciences, Alagappa University, Thondi Campus, Thondi – 623 409, Ramanathapuram District, Tamil Nadu, India; ²Division of Algal Biotechnology, Department of Botany, Annamalai University, Annamalainagar, Chidambaram, Tamil Nadu, India;

*Correspondence: sandalsrini@gmail.com; Tel.: +91 9788780266

Abstract: The present study was carried out to determine the influence of ecological characteristics and bioluminescent bacterial distribution in the seawater and sediment of Palk Strait, Southeast coast of India during July 2010 - June 2012. The physico-chemical parameters such as., atmospheric temperature range was varied from 24.3 - 35.3°C, surface water temperature 23.2 - 33.5°C, pH (6.2 - 8.91), dissolved oxygen concentration ranged from 3.84 - 7.73ml l⁻¹, salinity fluctuated between 23.12 and 39 ppt, POC concentration ranged from 0.78 - 2.56mg dry wt.l⁻¹. The seawater was analysed and results suggest that it contains, inorganic phosphate (4.12 - 21.6µM), reactive silicate (4.3 - 19.26µM), inorganic nitrate (1.95 - 12.25µM), inorganic nitrite (1.1 - 5.5µM), total nitrogen (1.5 - 10.39µM), calcium (120 - 990 mg l⁻¹) and magnesium (730 - 2460mg l⁻¹). Bottom water temperature ranged from 20.4 to 27.1°C, sediment temperature (17 to 25°C), sediment pH (6.2 to 9.1), concentration of sediment nutrients and heavy metals phosphorus concentration ranged from 0.065 to 0.315%, potassium (3.18 to 8.57%), calcium (4.08 to 25.44%), magnesium (0.29 to 1.7%), silicon (33.2 to 56.53%), sodium (1.56 to 3.35%), sulphur (0.32 to 2.06%), chloride (1.3 to 5.71%), aluminium (4.63 to 11.87%), titanium (1.55 to 9.15%), manganese (0.08 to 0.26%), iron (3.2 to 11.13%), cobalt (3 to 11ppm), copper (4 to 29ppm), chromium (26 - 85ppm), nickel (5 to 18ppm) and lead (11 to 27ppm). Seawater colony forming unit (CFU) for the bioluminescent bacterial population density was varied from 1.06 x 10⁴ to 9.44 x 10⁴ CFU/ml and sediment (2.6 x10⁴ to 23.2 x10⁴ CFU/g). Statistical analysis seawater colony forming unit (CFU) of bioluminescent bacteria showed a positive correlation with salinity and water pH. Sediment colony forming unit of bioluminescent bacteria showed a positive correlation sediment temperature, sediment pH, sediment silicon, sodium and chloride. It can be concluded that the ecological parameters were observed in water and sediment which highly influence the bioluminescent bacterial populations and their diversity.

Keyword: Bioluminescent bacteria; colony formation Palk Strait; diversity; physico-chemical parameters

1. Introduction

Bioluminescence is a ubiquitous feature of the world oceans and originates from organisms representing all tropic levels. They are ecologically versatile,

present in water, sediment and also harbored in the light organs of some fish and gut of many marine organisms (Hastings, 1986; Govindasamy and Srinivasan, 2012). Bioluminescence is also subject to control by a number of environmental

factors. Low oxygen tension may increase luminescence and luciferase levels (Ruby and Nealson, 1978; Nealson and Hastings, 1979). Although on large scales bioluminescence may be correlated with hydrographic features (Swift *et al.*, 1995). The marine environment contains an immense diversity of microbes that have evolved to perform equally diverse functions in their respective environments and ecological niches. Particularly when their populations are dense, disturbance of the water during the night causes bright blue bioluminescent display that have been reported (Harvey, 1952) and are now known to occur globally (Lynch, 1981). Its widespread distribution, bioluminescence is clearly a predominant form of communication in the sea, with important effects on the immense daily vertical migration, predator-prey interactions and the flow of material through the food web. Bioluminescent bacteria emit light continuously, whereas higher organisms usually emit light in pulses, accomplished by localizing the systems into organelles that are regulated by pH change, calcium flux and oxygen (Nelson and Hastings, 1979). These are among the most extensively studied group of marine bacteria with regard to ecology, taxonomy and phylogeny. Many studies have suggested that the distribution and species composition of luminous bacteria are influenced by season, temperature, nutrient concentration, depth and geographical location (Nair *et al.* 1979; Ramaiah and Chandramohan, 1988). Furthermore, the chemical reaction, which produces light in bioluminescent bacteria, is essentially the same for all species (Nealson and Hastings, 1979).

In spite of the fact that a wide range of habitats are occupied by luminous bacteria, very little information concerning their distribution of luminous bacteria in Palk Strait, coastal region especially in seagrass meadows sediments. Hence, the present study was undertaken to understand the ecology, distribution and diversity of bioluminescent bacteria in this seagrass ecosystem along with en-

vironmental parameters in Devipattinam, Thondi and Manamelkudi region of Palk Strait, Southeast coast of India.

2. Materials and methods

A total of 144 water and sediment samples were collected from three different places of Palk Strait viz., Devipattinam (Station I; Lat. 9°28'N; Long. 78°54'E), Thondi (Station II; Lat. 9°45'N; Long. 79°3'E) and Manamelkudi (Station III; Lat. 10°3'N; Long. 79°13'60'E) during July 2010 - June 2012. The surface water sample was collected with sterile polypropylene bottle and sediment sample were also collected using alcohol-rinsed, air dried, Peterson grab sampler. The central portion of the top 2cm sediment samples was taken out with the help of a sterile spatula and the samples were then transferred into a sterile polythene bag. All samples were transported to the laboratory within 1-3 hours of collection under ice cold condition.

Atmospheric, surface water, bottom water and sediment temperatures were measured using standard mercury filled centigrade thermometer. Salinity was estimated with the help of hand refractometer (Model 2491 Master-S/Milla). Seawater pH was measured using high configuration digital pH pen. Soil pH was measured at a soil/water ratio of 1:2.5 (w/w). Air-dried soil (10g, 2.8 mm) and 25 ml distilled water were shaken together for 2 min and left to settle for 30 min, this procedure was repeated once and then the pH was determined using high configuration digital pH pen (Model No: Reed 8690; ± 0.001) (Rousk *et al.*, 2009). Dissolved Oxygen, Particulate Organic Carbon (POC), inorganic Phosphate (PO_4), reactive Silicate (SiO_3), inorganic Nitrate (NO_3), inorganic Nitrite (NO_2), Total Nitrogen (TN), and Calcium (Ca) and Magnesium (Mg) were analyzed as described by Parsons *et al.* (1984). All sediment samples were air-dried at 25°C in the laboratory of Department of Oceanography and Coastal Area Studies, Alagappa Uni-

versity, India. The soil particles were disaggregated, crushed and sieved through 10mm nylon sieve and then stored in polythene bags and labelled at 4°C until the analysis. All the elemental present in the sediments were analyzed by using Bruker™ S4-Pioneer model wavelength dispersive X-ray fluorescence spectrophotometer (WD-XRF). The samples were ground to particle size well below 100µm using ball mill in order to minimize the grain size interference on XRF-measurement. The major elements viz., Phosphorus (P), Potassium (K), Calcium (Ca), Magnesium (Mg), Silicon (Si), Sodium (Na), Sulphur (S), Chloride (Cl), Aluminium (Al), Titanium (Ti), Manganese (Mn), Iron (Fe), Cobalt (Co), Copper (Cu), Chromium (Cr), Nickel (Ni) and Lead (Pb) were analyzed.

Then, 1ml seawater/ 1g sediments of samples were mixed with 99/100ml of 50% aged sterile seawater mixed vigorously and used for serial dilution in test tubes upto 10^{-2} dilutions up to 10^{-7} . About 0.1ml from the each dilution was spread in luminescent agar (LA) medium (Dehydrated Nutrient broth 8g, Sodium Chloride 30g, Glycerol 3g, Calcium Carbonate

5g, Agar 15g, Seawater 1000ml, pH 7.2 ± 0.1). The plates were incubated at 32°C for 24 hrs. After incubation, the colonies were counted in the dark room and expressed in CFU ml/g and identified by the standard procedure (Nealson, 1978). The isolated bioluminescent colonies were purified and stored at 4°C with 40% glycerol until analysis (Kita-Tsukamoto *et al.*, 2006). All the data were computed using SPSS v 16.0 statistical software to obtain Pearson's correlation co-efficient (r) for the statistical interpretation. The correlation coefficient and standard deviation (SD $\pm$) were calculated between the physico-chemical variables and CFU of bioluminescent bacterial population in all samples collected from all the stations from June 2010 to May 2012.

3. Results

Atmospheric temperature was varied from 24.3 to 35.3°C (Figure 1), the surface water temperature was varied from 23.2 to 33.5°C (Figure 2). The pH range was from 6.2 to 8.91 (Figure 3), the

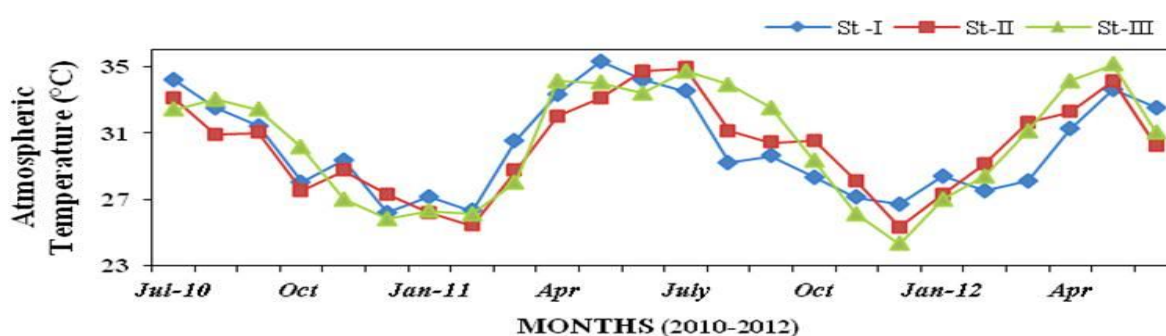


Figure 1: Monthly variations of atmospheric temperature (°C) recorded at stations I, II and III from July 2010 to June 2012.

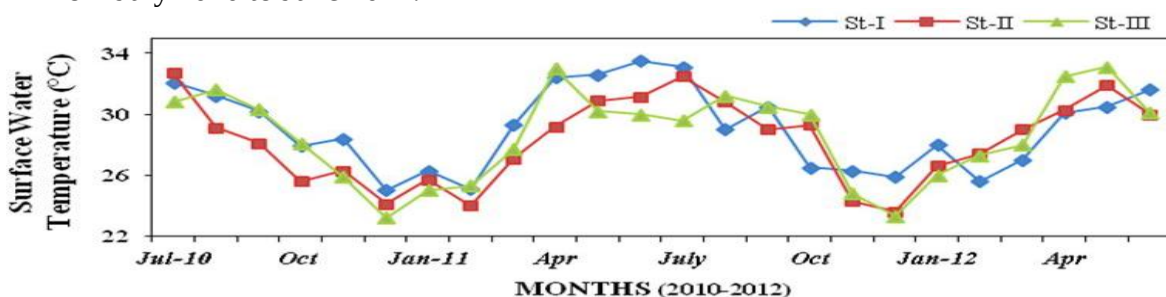


Figure 2: Monthly variations of surface water temperature (°C) recorded at stations I, II and III from July 2010 to June 2012.

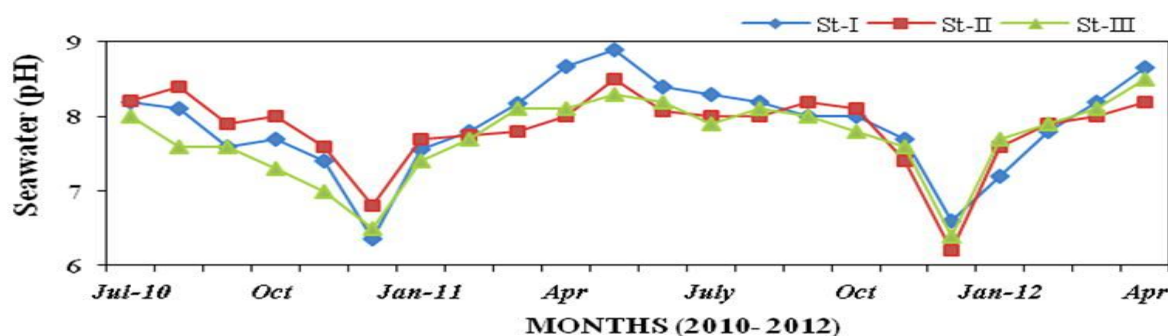


Figure 3: Monthly variations of hydrogen ion concentration (pH) in seawater recorded at stations I, II and III from July 2010 to June 2012.

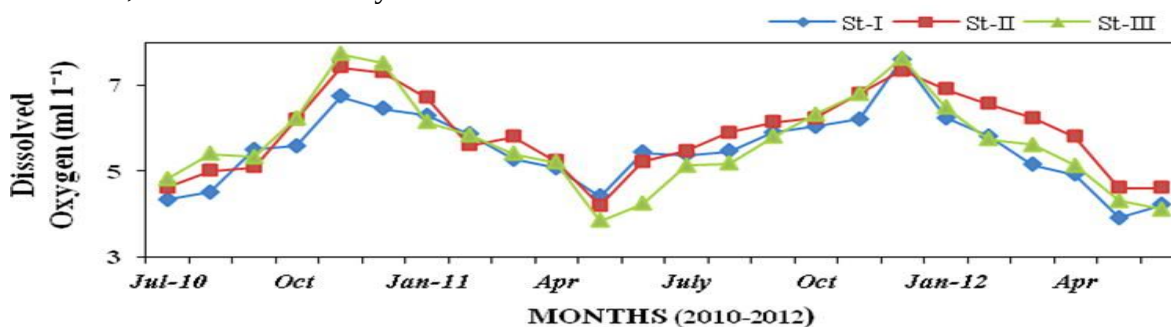


Figure 4: Monthly variations of dissolved oxygen (ml l^{-1}) recorded at stations I, II and III from July 2010 to June 2012.

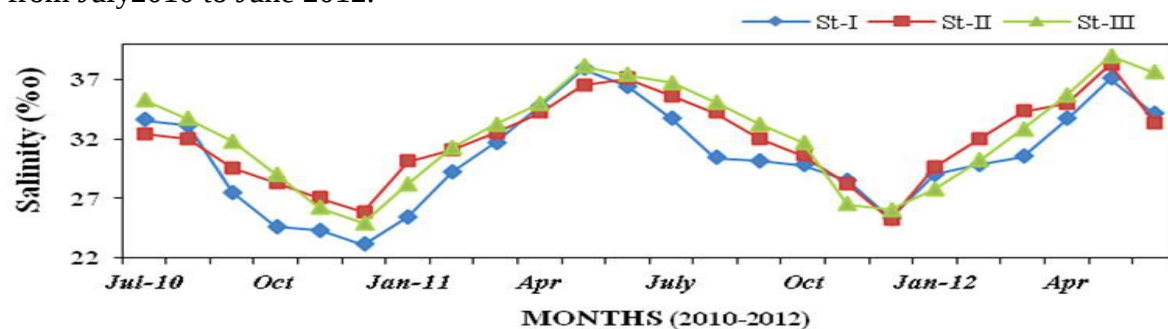


Figure 5: Monthly variations of salinity ($\text{S}_{\text{‰}}$) in seawater recorded at stations I, II and III from July 2010 to June 2012.

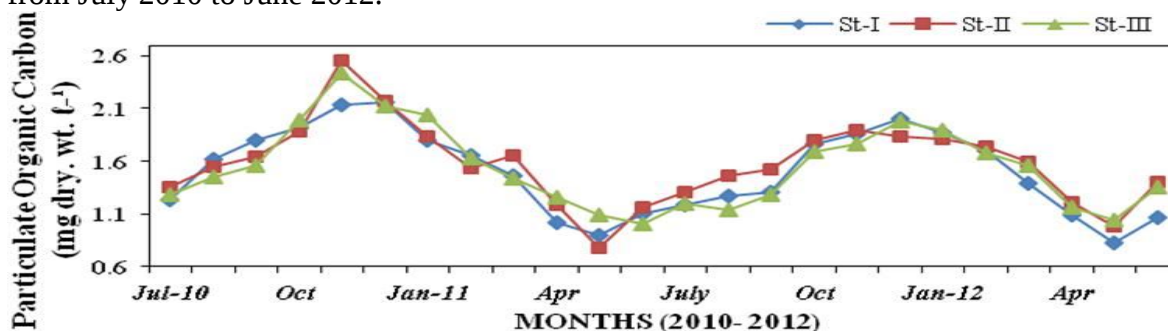


Figure 6: Monthly variations of particulate organic carbon (mg dry wt. l^{-1}) recorded at stations I, II and III from July 2010 to June 2012.

dissolved oxygen concentration ranged from 3.84 to 7.73 ml l^{-1} (Figure 4) and the salinity was fluctuated between 23.12 and 39 ppt (Figure 5). Particulate organic carbon concentration ranged from 0.78 to $2.56 \text{ mg dry wt. l}^{-1}$ during the study period at all the stations (Figure 6). The results

showed that seawater contains nutrients viz., inorganic phosphate (4.12 to $21.6 \mu\text{M}$; Figure 7), reactive silicate (4.3 to $19.26 \mu\text{M}$; Figure 8), inorganic nitrate (1.95 to $12.25 \mu\text{M}$; Figure 9), inorganic nitrite (1.1 to $5.5 \mu\text{M}$; Figure 10), total nitrogen (1.5 to $10.39 \mu\text{M}$; Figure 11),

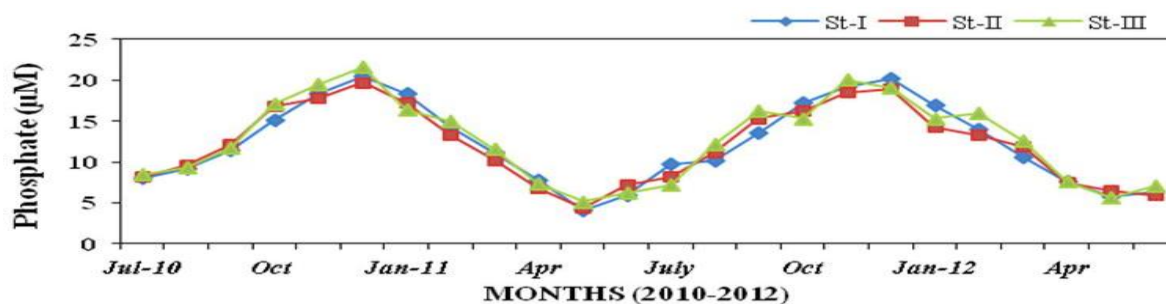


Figure 7: Monthly variations of inorganic phosphate (μM) concentration in seawater recorded at stations I, II and III from July 2010 to June 2012.

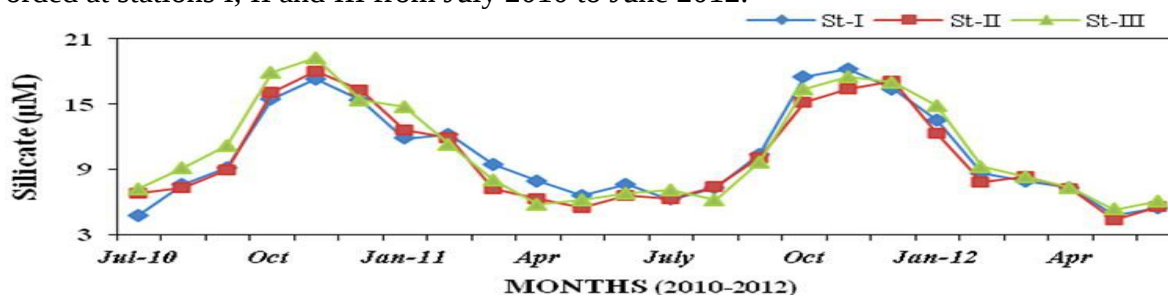


Figure 8: Monthly variations of reactive silicate (μM) concentration in seawater recorded at stations I, II and III from July 2010 to June 2012.

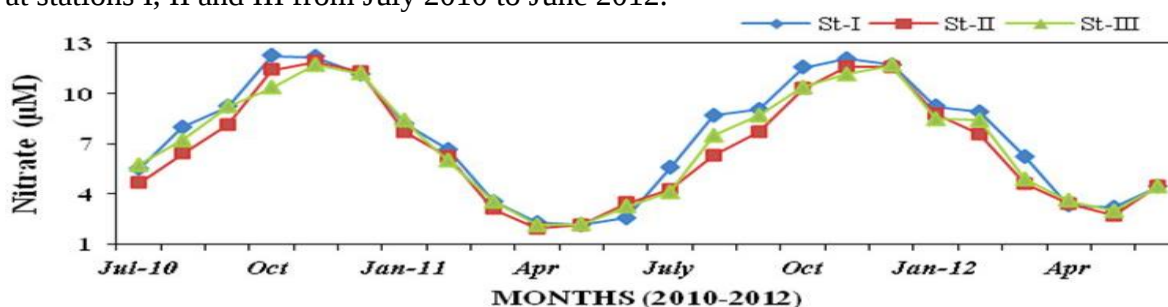


Figure 9: Monthly variations of inorganic nitrate (μM) concentration in seawater recorded at stations I, II and III from July 2010 to June 2012.

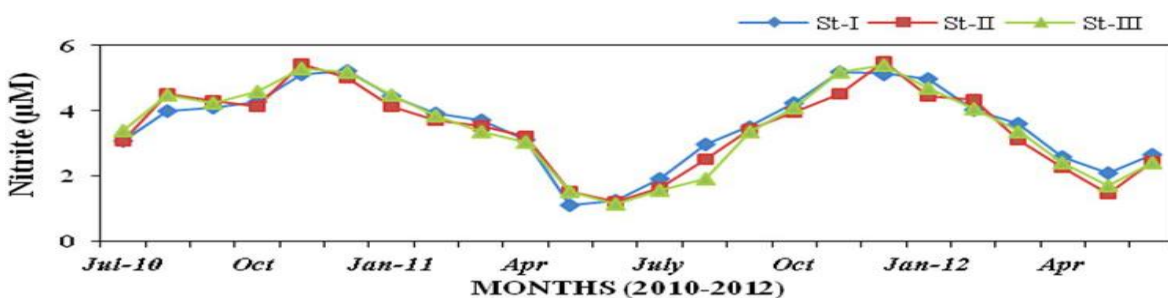


Figure 10: Monthly variations of inorganic nitrite (μM) concentration in seawater recorded at stations I, II and III from July 2010 to June 2012.

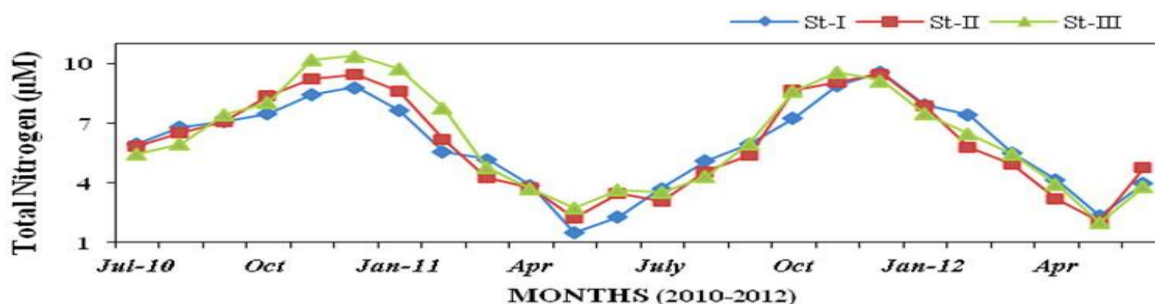


Figure 11: Monthly variations of total nitrogen (μM) concentration in seawater recorded at stations I, II and III from July 2010 to June 2012.

calcium (120 to 990 mg l⁻¹; Figure 12) and magnesium (730 to 2460mg l⁻¹; Figure13). The bacterial diversity revealed that, the bioluminescent bacterial population density was varied from 1.06 x 10⁴ to 9.44 x 10⁴ CFU/ml in seawater (Figure 14).

Bottom water temperature was in the range of 20.4 to 27.1°C during the study period at all three stations (Figure 15). Sediment temperature varied from 17 to 25°C (Figure 16). Sediment pH varied from 6.2 and 9.1 at all stations (Figure 17). The results for sediment nutrients showed that phosphorus (0.065 to 0.315%; Figure 18), potassium (3.18 to 8.57%; Figure 19), calcium (4.08 to

25.44%; Figure 20), magnesium (0.29 to 1.7%; Figure21), silicon (33.2 to 56.53%; Figure 22), sodium (1.56 to 3.35%; Figure 23), sulphur (0.32 to 2.06%; Figure 24), chloride (1.3 to 5.71%; Figure 25), aluminium (4.63% to 11.87%; Figure 26), titanium (1.55 to 9.15%; Figure 27), manganese (0.08 to 0.26%; Figure28), iron (3.2 to 11.13%; Figure 29), cobalt (3 to 11ppm; Figure 30), copper (4 to 29ppm Figure 31), chromium (26 to 85ppm; Figure 32), nickel (5 to 18ppm; Figure 33) and lead (11 to 27ppm; Figure 34). In sediment, the bioluminescent bacterial population density varied from 2.6 x10⁴ to 23.2 x10⁴ CFU/g (Figure 35).

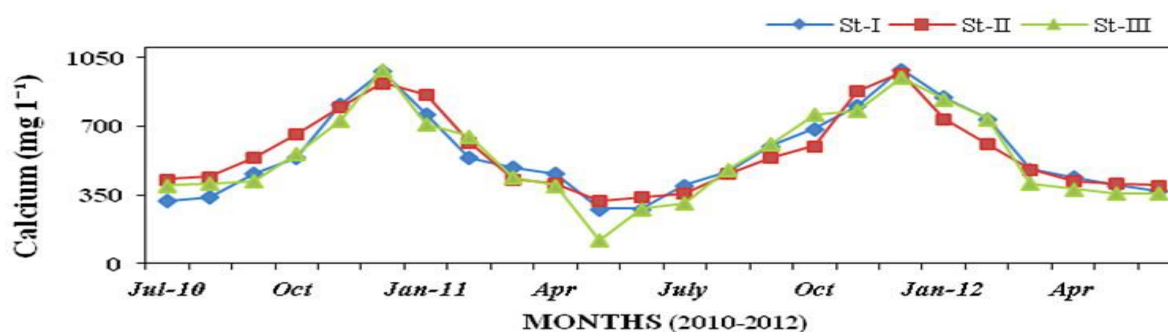


Figure 12: Monthly variations of calcium (mg l⁻¹) concentration in seawater recorded at stations I, II and III from July 2010 to June 2012.

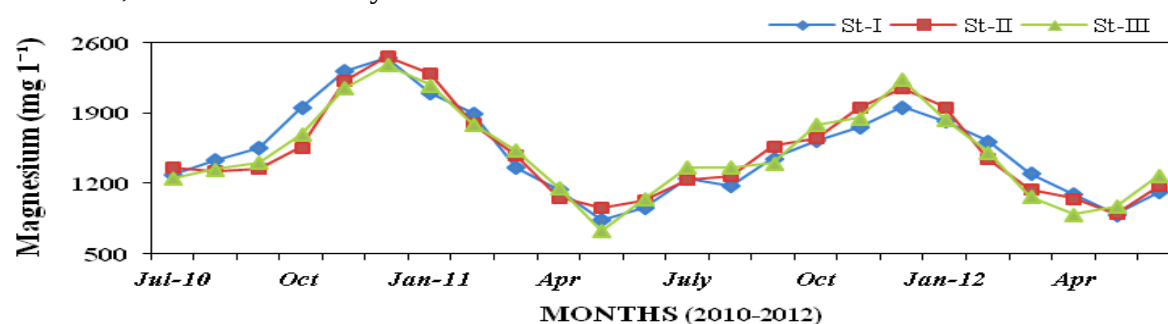


Figure 13: Monthly variations of magnesium (mg l⁻¹) concentration in seawater recorded at stations I, II and III from July 2010 to June 2012.

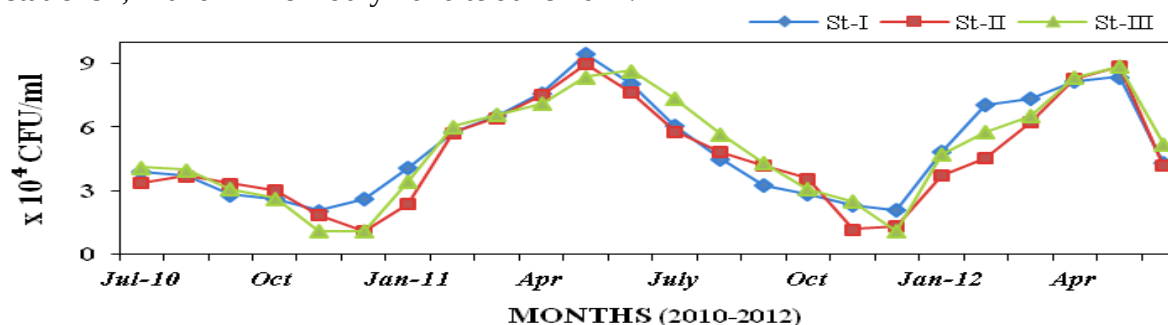


Figure 14: Monthly variations of bioluminescent bacteria (CFU/ml x 10⁴) populations in seawater recorded at stations I, II and III from July 2010 to June 2012.

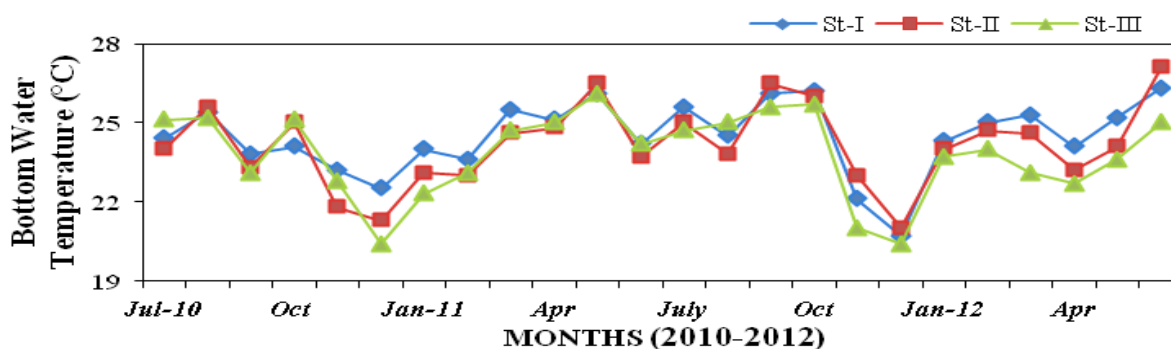


Figure 15: Monthly variations in bottom water temperature (°C) recorded at stations I, II and III from July 2010 to June 2012.

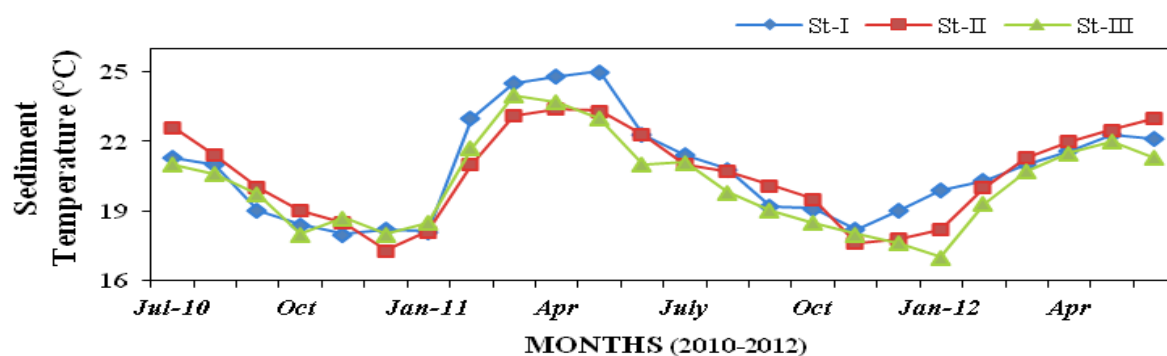


Figure 16: Monthly variations in sediment temperature (°C) recorded at stations I, II and III from July 2010 to June 2012.

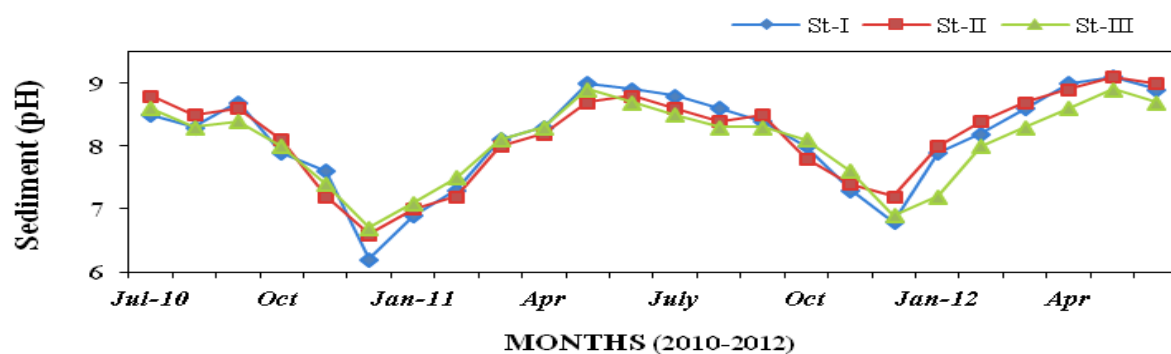


Figure 17: Monthly variations of hydrogen ion concentration (pH) in sediment recorded at stations I, II and III from July 2010 to June 2012.

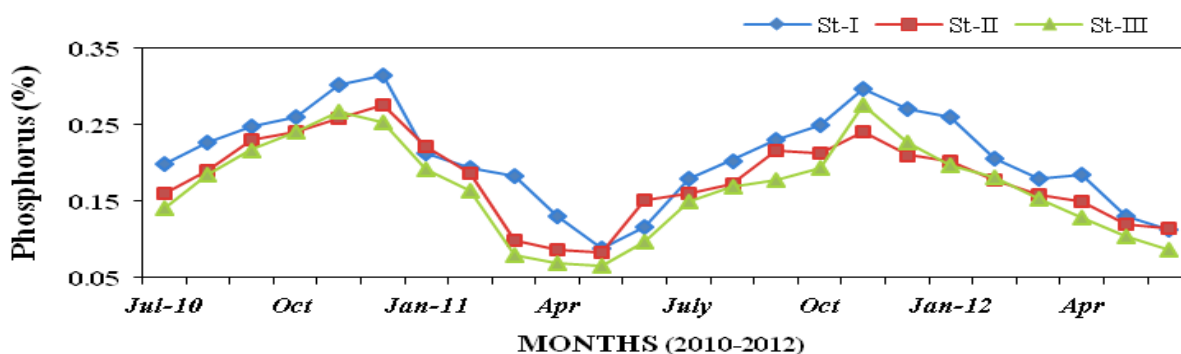


Figure 18: Monthly percentage variations of phosphorus (%) concentration in sediment recorded at stations I, II and III from July 2010 to June 2012.

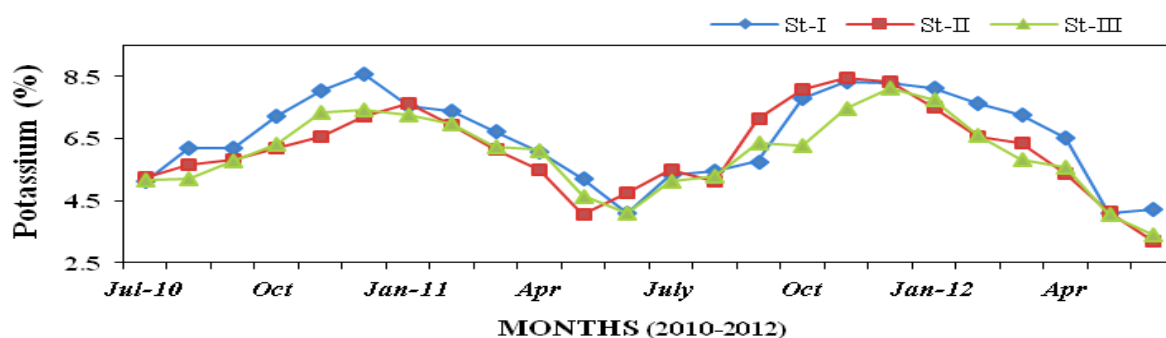


Figure 19: Monthly percentage variations of potassium (%) concentration in sediment recorded at stations I, II and III from July 2010 to June 2012.

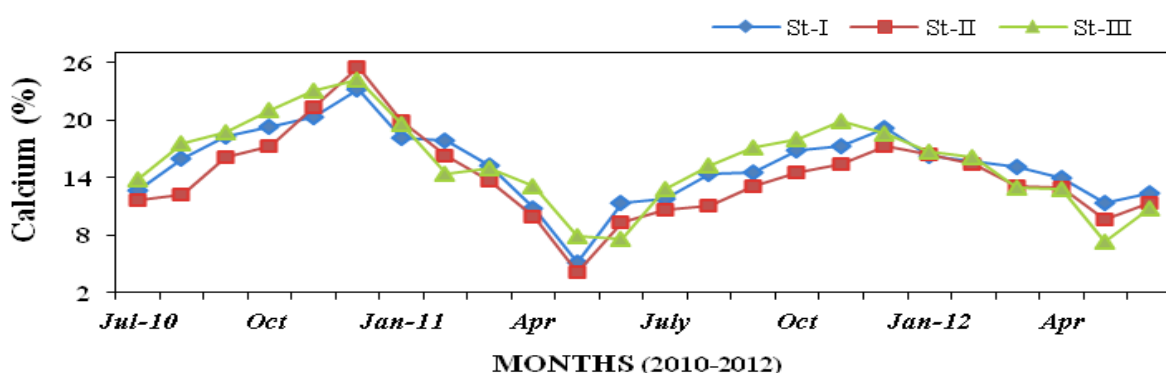


Figure 20: Monthly percentage variations of calcium (%) concentration in sediment recorded at stations I, II and III from July 2010 to June 2012.

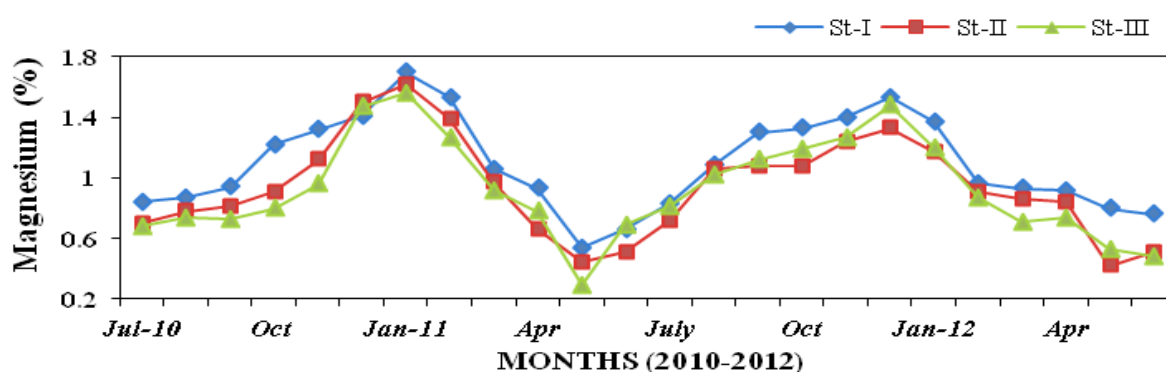


Figure 21: Monthly percentage variations of magnesium (%) concentration in sediment recorded at stations I, II and III from July 2010 to June 2012.

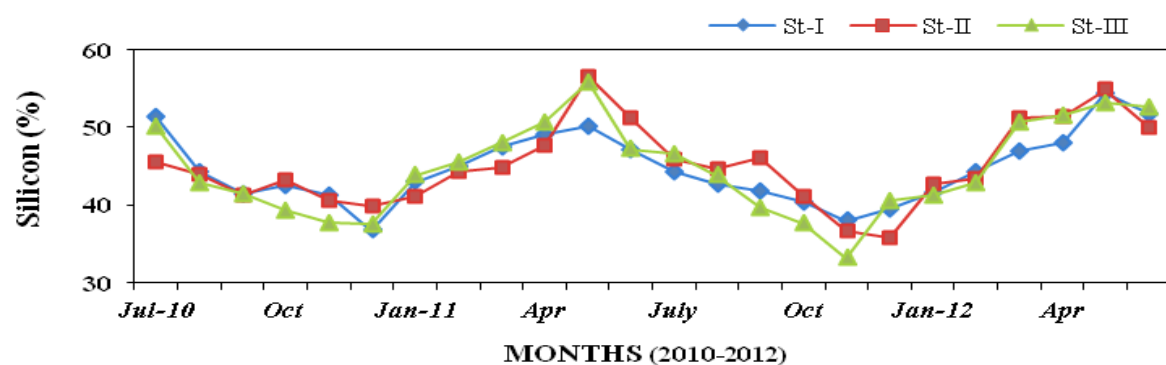


Figure 22: Monthly percentage variations of silicon (%) concentration in sediment recorded at stations I, II and III from July 2010 to June 2012.

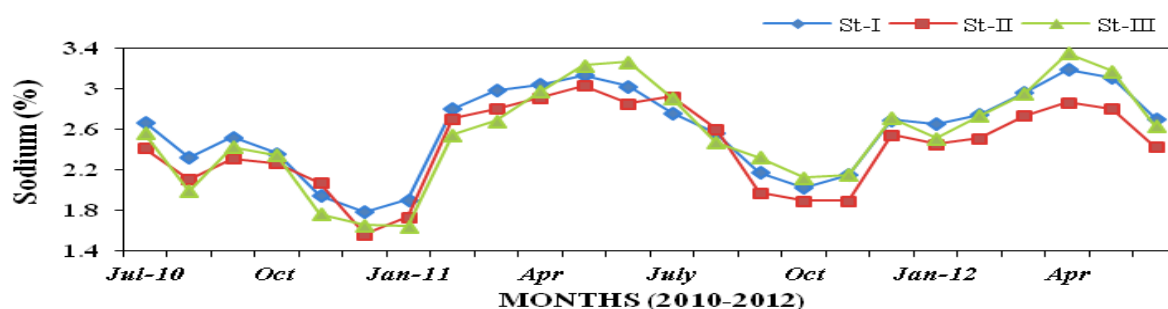


Figure 23: Monthly percentage variations of sodium (%) concentration in sediment recorded at stations I, II and III from July 2010 to June 2012.

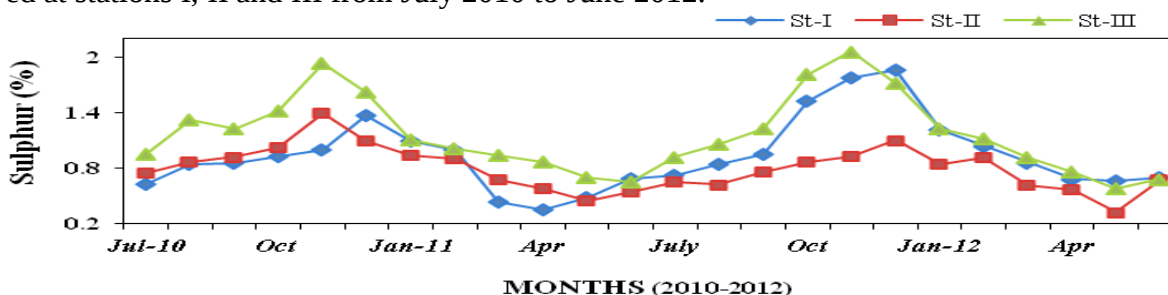


Figure 24: Monthly percentage variations of sulphur (%) concentration in sediment recorded at stations I, II and III from July 2010 to June 2012.

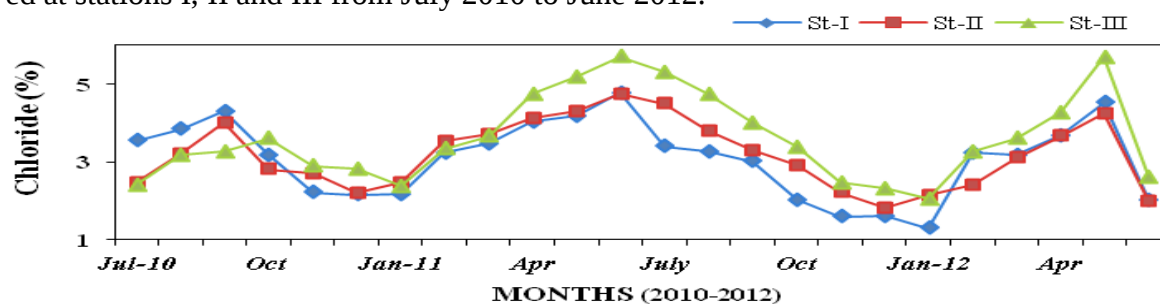


Figure 25: Monthly percentage variations of chloride (%) concentration in sediment recorded at stations I, II and III from July 2010 to June 2012.

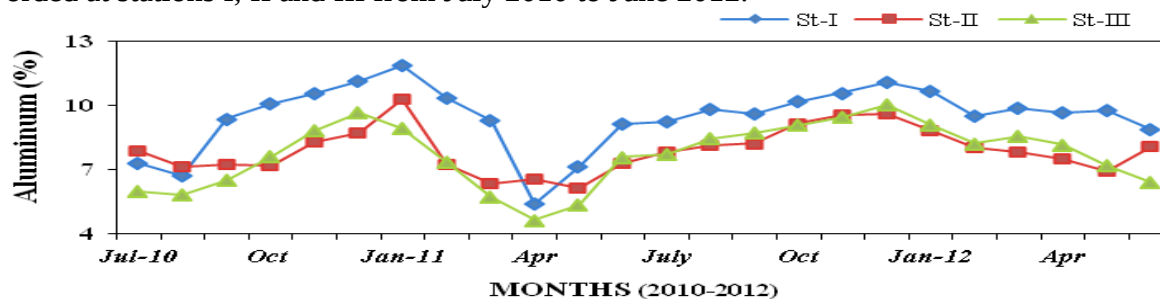


Figure 26: Monthly percentage variations of aluminum (%) concentration in sediment recorded at stations I, II and III from July 2010 to June 2012.

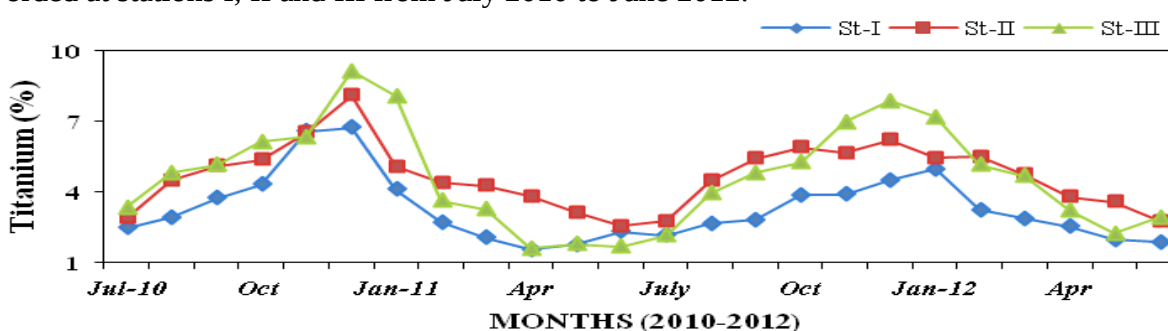


Figure 27: Monthly percentage variations of titanium (%) concentration in sediment recorded at stations I, II and III from July 2010 to June 2012.

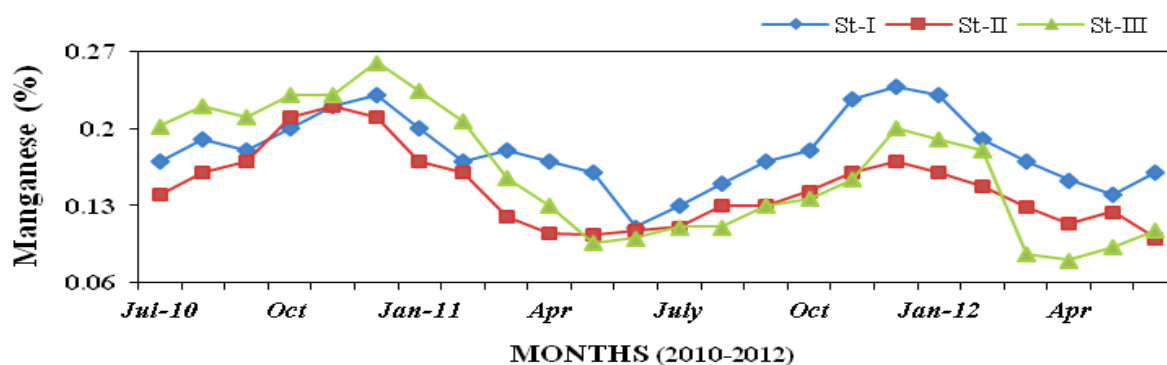


Figure 28: Monthly percentage variations of manganese (%) concentration in sediment recorded at stations I, II and III from July 2010 to June 2012.

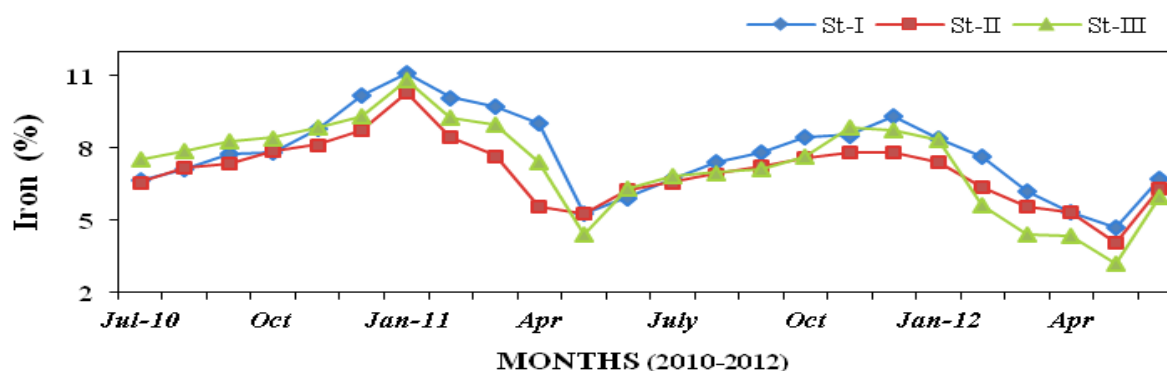


Figure 29: Monthly percentage variations of iron (%) concentration in sediment recorded at stations I, II and III from July 2010 to June 2012.

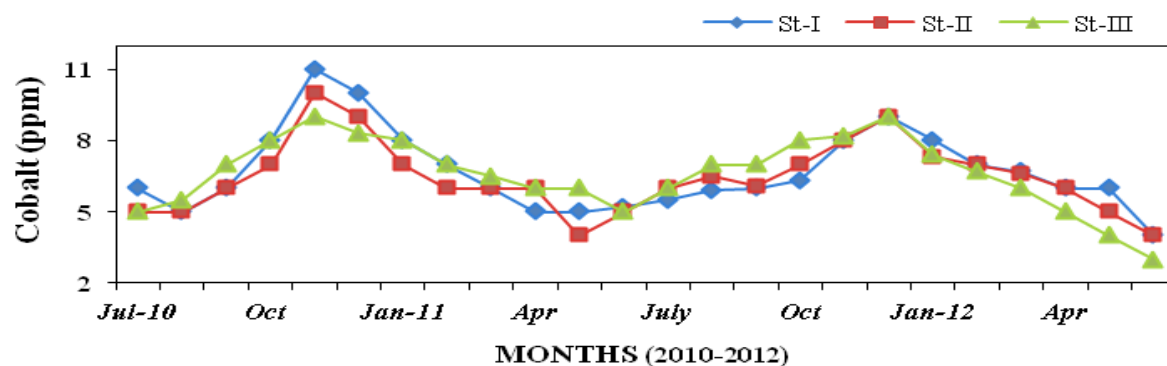


Figure 30: Monthly variations of cobalt (ppm) concentration in sediment recorded at stations I, II and III from July 2010 to June 2012.

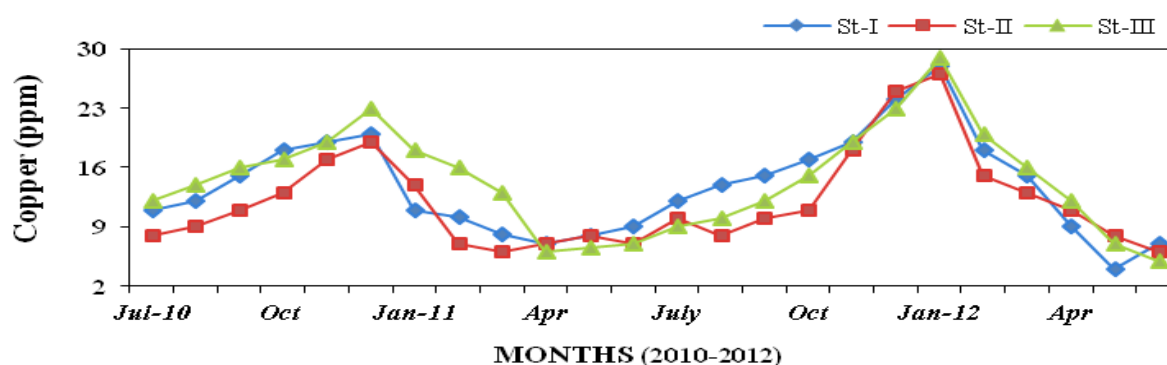


Figure 31: Monthly variations of copper (ppm) concentration in sediment recorded at stations I, II and III from July 2010 to June 2012.

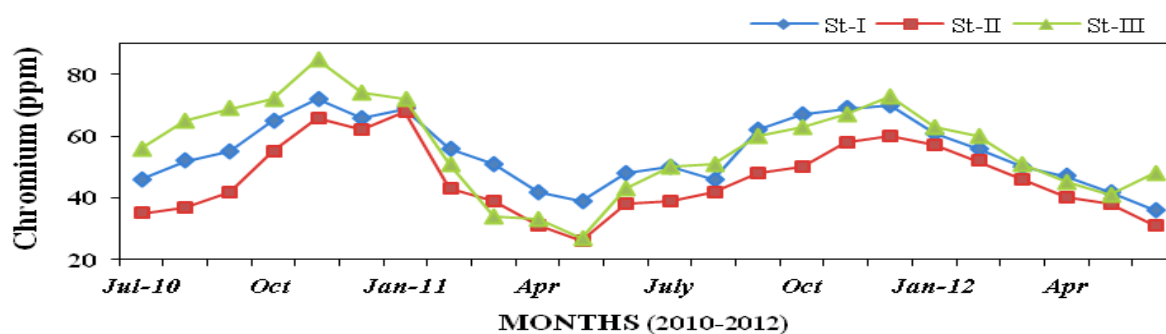


Figure 32: Monthly variations of chromium (ppm) concentration in sediment recorded at stations I, II and III from July 2010 to June 2012.

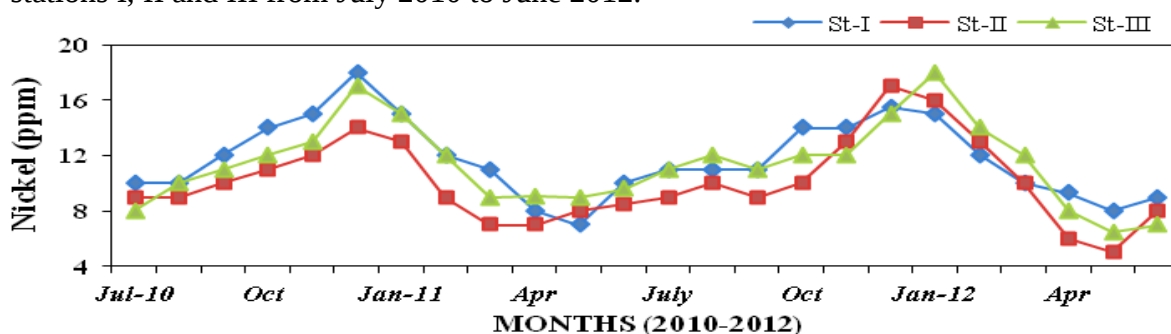


Figure 33: Monthly variations of nickel (ppm) concentration in sediment recorded at stations I, II and III from July 2010 to June 2012.

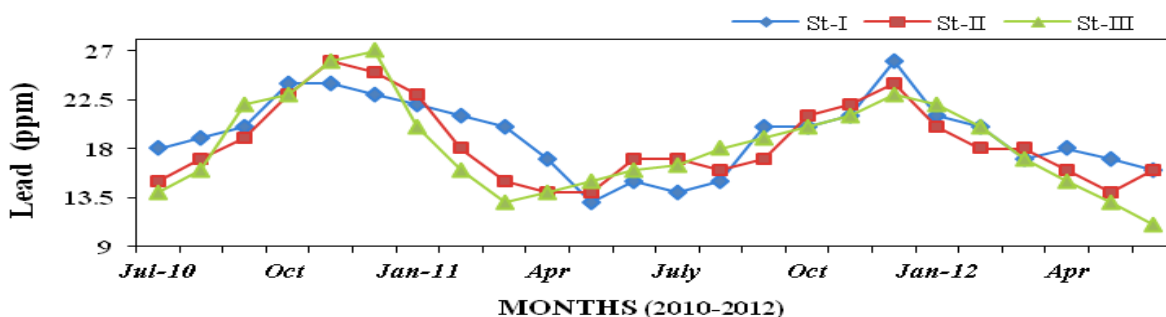


Figure 34: Monthly variations of lead (ppm) concentration in sediment recorded at stations I, II and III from July 2010 to June 2012.

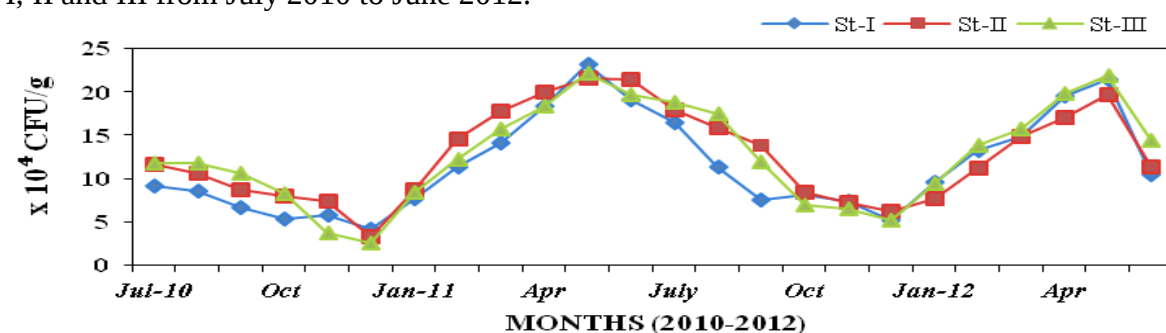


Figure 35: Monthly variations of bioluminescent bacteria (CFU/g $\times 10^4$) populations in sediment recorded at stations I, II and III from July 2010 to June 2012.

4. Discussion

Based on the results, the physico-chemical parameters, heavy metals in water and sediments would form a useful

tool for the assessment and monitoring of coastal ecosystems. The similar results and trend was observed in Muthupattai mangroves, Southeast coast of India (Ashokkumar *et al.*, 2009; Senthilnathan

and Balasubramanian, 1999). The higher concentration of metals were recorded during monsoon season, which could be mainly due to land runoff and influx of metal rich freshwater that in turn reflects in the metal concentration in sediment (Athalye and Gokhale, 1989). The assessment of trace metal concentrations and distribution in marine water and sediment leads to an understanding of their behaviour and detects the pollution source in marine environment (Forstner and Wittman, 1979). Besides substantiating higher biological productivity, higher densities of luminous bacteria in this bay also signify pollution free environment in this region.

Bioluminescent bacteria are being found in marine environment. Microbial activities play an important role in marine food webs, nutrient mineralization and recycling. The ecological importance of bioluminescence in the ocean is evident in the dominance of light emitters in open waters. Ecology of bioluminescent bacteria has focused primarily on distribution of these organisms in marine environment (Nealson and Hastings, 1979); Atlantic Ocean (Ramaiah and Chandramohan, 1988), Indian Ocean (Lynch, 1981) and near shore water Porto Nova (Ramesh *et al.*, 1989).

The present study was carried out to understand the role of ecological parameters of the bioluminescent bacteria (during July 2010 - June 2012) at different stations of Palk Strait region, India. The maximum counts of bioluminescent bacteria in seawater/ sediments samples was recorded during summer season (May 2011) at station I; whereas, minimum counts of bioluminescent bacteria seawater/ sediment samples was recorded at monsoon season (2010) at station III. The CFU values suggested that the higher population counts were recorded during the summer months during the study periods at all the stations. This variation might be due to the high-saline relativity. The statistical analysis revealed that seawater colony forming unit (CFU) of bio-

luminescent bacteria showed a positive correlation with salinity at station-I ($r=0.773$; $P<0.001$), station-II ($r=0.903$; $P<0.001$) and station-III ($r=0.837$; $P<0.001$) and water pH at station-I ($r=0.726$; $P<0.01$), station-II ($r=0.631$; $P<0.01$) and station-III ($r=0.874$; $P<0.001$). The counts were at low levels during the active monsoon period because of high rainfall. The monsoonal flood water may have altered the luminous bacterial population from sediment as the Vellar estuary is shallow (Ramesh *et al.*, 1989). Further, the maximum atmospheric, surface water and sediment temperature were recorded during summer at station I and minimum was recorded during monsoon at station III. Maximum bottom water temperature was recorded during pre-monsoon at station I and minimum was recorded during monsoon at station III. Surface water temperature was slightly higher than the bottom water at all the stations. Surface and bottom water temperature of all stations are slightly varied monthly. Temperature is an important environmental factor, can influence the diversity of luminous bacteria (Ruby and Nealson, 1978; Yetinson and Shilo, 1979; Ruby *et al.*, 1980). Dunlap (2009) reported that the temperature relationships of luminous bacteria appear to be a specific to *Vibrio* species. According to Govindasamy *et al.* (2000), the surface water temperature could be changed by the intensity of solar radiation; evaporation, freshwater influx, cooling and it might mix up with ebb and flow from adjoining neritic water. It is further evident that the atmospheric temperature showed positive correlation at station-II ($r=0.663$; $P<0.01$) and at station - III ($r=0.685$; $P<0.01$) to seawater with CFU microbial counting at all the stations. Surface water temperature was found low during monsoon because of rainfall but the maximum temperature was observed during summer (Kannapiran *et al.*, 2008). This could be attributed due to high solar radiation as reported by Ashok Prabu *et al.* (2008). Lower temperature was observed due to cloudy sky and rainfall that brought down

temperature to minimum (Kannan and Kannan, 1996). In addition, the surface water temperature showed positive correlation with colony forming unit (CFU) at station - II ($r = 0.635$; $P < 0.02$) and at station - III ($r = 0.627$; $P < 0.02$). Further, the statistical analysis revealed that the sediment colony forming unit of bioluminescent bacteria showed a positive correlation with sediments temperature at station - I ($r = 0.806$; $P < 0.001$), station-II ($r = 0.842$; $P < 0.001$) and station-III ($r = 0.784$; $P < 0.001$).

Surface water salinity was reduced greatly during the monsoon and it was gradually increased from postmonsoon to summer at all stations. The maximum salinity could be due to low amount of rainfall and higher rate of evaporation in the shallow coastal area owing to high atmospheric temperature (Govindasamy and Kannan, 1991). Significantly positive correlation was observed between seawater salinity and CFU of bioluminescent bacteria at station-I ($r = 0.773$; $P < 0.001$), station-II ($r = 0.903$; $P < 0.001$) and station-III ($r = 0.837$; $P < 0.001$). The present study results are in line with the research findings of Abraham *et al.* (2003).

The high pH values recorded during summer and this might be due to the influence of seawater penetration and high biological activity. These findings are in accordance with the previous report (Smith and Key, 1975). The statistical analysis shows the positive correlation between pH and CFU station - I ($r = 0.726$; $P < 0.01$) and station - III ($r = 0.874$; $P < 0.001$) to seawater colony forming unit. The statistical analysis shows the positive correlation with sediments pH station-I ($r = 0.692$; $P < 0.001$), station-II ($r = 0.641$; $P < 0.001$) and station-III ($r = 0.813$; $P < 0.001$) to sediment colony forming unit. Dissolved oxygen is one of the most important abiotic parameters influencing the life in the coastal environment. In the present study, the maximum dissolved oxygen was recorded during monsoon and this might be due to the cumulative effect of higher wind velocity coupled with rain-

fall and the resultant freshwater mixing. The minimum dissolved oxygen was found during summer months, which could be mainly due to reduced agitation in the coastal and estuarine water (Ruby and Neilson, 1978; Neilson and Hasting, 1979). Further, this is evidenced by the negative correlation between the dissolved oxygen and seawater CFU at station-III ($r = -0.823$; $P < 0.001$).

Maximum particular organic carbon (POC) was recorded during the month of November (2010) at station-II and minimum POC was observed during the month of May (2012) at station-I. The maximum POC content in water was mainly due to the organic matter brought in from the land through run-off. Further, it could be also due to the presence of plant (seagrass and seaweeds) and animal organic matter within the seagrass ecosystem and exported from the adjacent ecosystem by wind and wave action. Further, the seasonal variation in POC content in the water could be related to the plankton productivity (Kannapiran *et al.*, 2008). This is further evidenced by the negative correlation between POC and seawater CFU at station-I ($r = -0.786$; $P < 0.001$), station-II ($r = -0.841$; $P < 0.001$) and station-III ($r = -0.832$; $P < 0.001$).

Maximum inorganic phosphate was observed during the monsoon season (December 2010) at station-III and minimum was recorded during the summer season (May 2011) at station-I. Possibly, the maximum concentration of phosphate was due to invasion of upwelling of water, which increased the level of phosphate. Low values of phosphate observed to utilization by phytoplankton, seagrasses and other primary producers (Rajasegar, 2003). The statistical analysis shows the negative correlation to seawater colony forming unit with inorganic phosphate at station - I ($r = -0.734$; $P < 0.01$), station-II ($r = -0.832$; $P < 0.001$) and station-III ($r = -0.828$; $P < 0.001$).

Minimum concentration of silicate was observed during the summer season (May 2012) at station II and maximum

during the monsoon season (November 2010) at station III. It might be due to the heavy rain, land runoff water mixing was high level. It has been reported that the silicate from the bottom sediment might have been exchanged with overlaying water in this mangrove environment (Govindasamy and Kannan, 1996). The low value observed in summer and post-monsoon season could be attributed to uptake of silicates (Saravanakumar *et al.*, 2008). The statistical analysis of silicate showed negative correlation with seawater colony forming unit at station-II ($r = -0.786$; $P < 0.001$) and station-III ($r = -0.841$; $P < 0.001$).

Nitrate concentration was found lower than that of nitrate and same trend of fluctuation was reported in Muthpettai mangrove (Ashokkumar *et al.*, 2011). The statistical analysis showed nitrate a negative correlation with seawater colony forming unit at station-I ($r = -0.868$; $P < 0.001$), station-II ($r = -0.888$; $P < 0.001$) and station-III ($r = -0.926$; $P < 0.001$).

Maximum level of nitrite was recorded during December 2011 at station-II and minimum was observed during the May 2011 at station-I. It might be due to the minimum nitrite were recorded during the summer may be due to high salinity. The higher nitrate value in monsoon season could be due to the increased phytoplankton excretion, oxidation of ammonia, reduction of nitrate, the recycling of nitrogen and bacterial decomposition of planktonic detritus (Govindasamy *et al.*, 2000; Asha and Diwakar, 2007) and also due to denitrification and air-sea interaction exchange of chemicals were also responsible for this increased values (Rajasegar, 2003; Ashok Prabu *et al.*, 2008). The statistical analysis which showed nitrite a negative correlation with seawater colony forming unit at station-I ($r = -0.767$; $P < 0.001$), station-II ($r = -0.834$; $P < 0.001$) and station-III ($r = -0.882$; $P < 0.001$).

Maximum level of total nitrogen was recorded during the monsoon (December 2010) at station III and minimum

was recorded during the summer (May 2011) at station I. It might be due to freshwater inflow was high in the monsoon season so high level of nitrogen was recorded. The statistical analysis showed total nitrogen a negative correlation with seawater CFU of bioluminescent bacteria at station-I ($r = -0.829$; $P < 0.001$), station-II ($r = -0.929$; $P < 0.001$) and station-III ($r = -0.896$; $P < 0.001$).

Maximum and minimum level of seawater calcium was observed during the monsoon (December 2010) and summer seasons (May 2011) at station-III. Maximum level of sediment calcium was observed during the monsoon season (December 2010) at station-II and minimum level of calcium was observed during the summer (May 2011) at station-II. Maximum level of seawater magnesium was recorded during the monsoon season December (2010) at station I and minimum was recorded during the summer season May (2011). Maximum level of sediment magnesium was observed during the post monsoon season (January 2011) at station-I and minimum level of magnesium was recorded during the summer season (May 2011) at station-II. The statistical analysis showed seawater calcium a negative correlation to seawater colony forming unit at station-II ($r = -0.816$; $P < 0.001$) and station-III ($r = -0.762$; $P < 0.001$). In addition, to that the seawater magnesium a negative correlation with seawater colony forming unit at station-II ($r = -0.829$; $P < 0.001$) and station-III ($r = -0.840$; $P < 0.001$). Sediment calcium a negative correlation with sediment colony forming unit at station-I ($r = -0.843$; $P < 0.001$), station-II ($r = -0.832$; $P < 0.001$) and station-III ($r = -0.909$; $P < 0.001$); Sediment magnesium showed a negative correlation with sediment colony forming unit at station-I ($r = -0.710$; $P < 0.001$), station-II ($r = -0.675$; $P < 0.001$) and station-III ($r = -0.721$; $P < 0.001$). It might be due to the calcium and magnesium higher level was recorded in monsoon and post monsoon seasons. Calcium and magnesium concentrations could be increased due to the in-

put of freshwater (Sundararajan and Natesan, 2010).

Maximum sediment total phosphorus was observed during the month of December-2010 at station-I and minimum was recorded during the month of May-2011 at station-III. and this might be due to the domestic, municipal and agricultural waste (non-pointed sources). The regeneration and releases of total phosphorus from bottom mud into the water column by turbulence and mixing was also attributed to recorded higher values (Chandran and Ramamoorthy, 1984). The statistical analysis showed sediment total phosphorus a negative correlation with sediments colony forming unit at station-I ($r = -0.848$; $P < 0.001$), station-II ($r = -0.843$; $P < 0.001$) and station-III ($r = -0.861$; $P < 0.001$). Maximum concentration of sediment potassium was observed during the monsoon season (December 2010) at station-I and minimum concentration of potassium was recorded during the summer (June 2010) at station-II. It might be due to high concentration of potassium are inflected by heavy rainfall inflow on in peak values was recorded during the monsoon seasons. Further, evidenced by the statistical analysis showed potassium a significant negative correlation with sediments colony forming unit at station-II ($r = -0.656$; $P < 0.001$) and station-III ($r = -0.751$; $P < 0.001$).

Maximum and minimum sediment silicon was observed during the May - 2012 at station II and November -2011 at station-III. This might be due to its biological significance; the flux of particulate silica from surface waters plays an important role in the cycling of other elements in the marine environment such as radium, barium and germanium (Li *et al.*, 1973; Froelich and Andreae, 1980). The statistical analysis showed silicon a significant positive correlation with sediment colony forming unit at station-I ($r = 0.745$; $P < 0.001$), station-II ($r = 0.824$; $P < 0.001$) and station-III ($r = 0.836$; $P < 0.001$). The statistical analysis shows sulphur showed a negative correlation with sedi-

ment colony forming unit at station-I ($r = -0.661$; $P < 0.001$), station-II ($r = -0.856$; $P < 0.001$) and station-III ($r = -0.882$; $P < 0.001$).

Maximum concentration of sediment sodium was observed during the month of 2011 at station-III and minimum was recorded at station-II and this might be due to the high counts of microbial populations depending on the sodium concentration and it was varied spatially. The statistical analysis which showed sediment sodium a positive correlation with sediment colony forming unit at station-I ($r = 0.834$; $P < 0.001$), station-II ($r = 0.824$; $P < 0.001$) and station-III ($r = 0.845$; $P < 0.001$). Maximum and minimum level of chloride was recorded during the summer and post monsoon seasons at station III and station I respectively. The statistical analysis of sediments chloride shows a positive correlation with sediment colony forming unit station-I ($r = 0.665$; $P < 0.001$), station-II ($r = 0.840$; $P < 0.001$) and station-III ($r = 0.811$; $P < 0.001$).

The maximum sediment aluminium was observed recorded during the post monsoon month of January 2012 at station-I and minimum was observed during the month of April 2011 at station-III. It might be due to contribution from detrital mineral grains supplied from through the rivers in addition to the precipitation of their dissolved species under prevailing estuarine condition. The statistical analysis which showed a negative correlation to sediment colony forming unit with aluminium at station-II ($r = -0.706$; $P < 0.001$). Removal of dissolved river-borne aluminum by coagulation in the coastal areas is common (Holliday and Liss, 1976).

In sediment, the maximum and minimum titanium level was recorded during the monsoon 2010 and summer April 2011 at stations III and I respectively. Moreover, the statistical analysis revealed that the sediment titanium shows a negative correlation to sediment colony forming unit at station-I ($r = -0.732$; $P < 0.001$), station-II ($r = -0.792$; $P < 0.001$)

and station-III ($r = -0.888$; $P < 0.01$). Maximum and minimum level of sediment manganese was recorded during the monsoon (2010) at station III and summer (2012) seasons at station I. The statistical analysis showed sediment manganese a negative correlation with sediment colony forming unit at station-I ($r = -0.732$; $P < 0.001$), station-II ($r = -0.819$; $P < 0.001$) and station-III ($r = -0.800$; $P < 0.001$).

Maximum and minimum concentration of sediment iron was recorded during the post monsoon (2011) and summer (2012) seasons at station I and III. In general, marine environment and discharge of aquatic ponds, domestic wastes, land and agricultural drainage, boating activities such as loading and unloading of materials, antifouling paints from boating activities contribute to enhance the metal level in marine environment (Govindasamy and Azariah, 1999; Ashokkumar *et al.*, 2009). The statistical analysis showed sediment iron a negative correlation with sediment colony forming unit at station-I ($r = -0.643$; $P < 0.01$), station-II ($r = -0.695$; $P < 0.001$) and station-III ($r = -0.758$; $P < 0.001$).

Maximum and minimum level of copper was observed during the month of January 2012 at station III and summer month of May (2012) at station I. It might be due to the discharge of maximum level of freshwater in the central west coast of India (Sankaranarayanan and Reddy, 1973). The statistical analysis showed sediment copper a negative correlation with sediment colony forming unit station-I ($r = -0.675$; $P < 0.001$), station-II ($r = -0.699$; $P < 0.001$) and station-III ($r = -0.770$; $P < 0.001$).

Maximum sediment chromium was recorded monsoon season (2010) at station III and minimum was recorded during summer season (2011) at station-II. It might be due to huge amount of freshwater river flow carry agricultural water, domestic sewage, animal and chromium in river-borne solids transported in estuaries is relatively higher than that of near shore sediments. Mannan *et*

al. (2004) has reported that effluent of metal finishing industry and corrosion of building materials play the main role to increase chromium level in marine environment. The statistical analysis showed sediment chromium a negative correlation with sediment microbial count at station-I ($r = -0.760$; $P < 0.001$), station-II ($r = -0.768$; $P < 0.001$) and station-III ($r = -0.896$; $P < 0.001$).

Maximum sediment nickel concentration was recorded during the monsoon (2010) at station I and minimum was recorded during the summer seasons (2012) at station II. The statistical analysis sediment nickel showed a negative correlation with sediment colony forming unit at station-I ($r = -0.801$; $P < 0.001$), station-II ($r = -0.778$; $P < 0.001$) and station-III ($r = -0.684$; $P < 0.001$). Maximum and minimum concentration of sediment lead was recorded during the monsoon (2010) and summer (June 2010) seasons at station-III. It might be due to the heavy rain river runoff and sewage discharges in the coastal region. Chatterjee *et al.* (2006) reported that 25.3 to 33.4mg/kg were observed in Hugi (Ganges) estuary, north-east coast of Bay of Bengal. The statistical analysis sediment lead showed a negative correlation with sediment colony forming unit station-I ($r = -0.778$; $P < 0.001$), station-II ($r = -0.835$; $P < 0.001$) and station-III ($r = -0.801$; $P < 0.001$).

5. Conclusion

The marine environment supports the survival, reproduction and the metabolic reactions of the flora and fauna. Among them, the microbial community plays a vital role in the food webs, biogeochemical cycles and the energy flow mechanisms in the marine ecosystem. But the microbial diversity in seagrass ecosystem is structured and determined by the prevailing temporal and spatial variability of ecological parameters in the native environment. The physico-chemical parameters of seawater were highly influencing the bioluminescent bacterial popu-

lation in the Palk Strait region. Most of the parameters such as the atmospheric temperature, salinity, pH were significantly correlated with the bioluminescent bacterial population in all the three stations studied.

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Synthesis of Biocompatible Silver Nanoparticles Using Green Alga (*Ulva reticulata*) Extract

Ganapathy Selvam Govindarasu¹, Srinivasan Rajendran² and Sivakumar Kathiresan^{1,*}

¹Division of Algal Biotechnology, Department of Botany, Annamalai University, Annamalai Nagar-608 002, Tamil Nadu, India; ²Department of Oceanography and Coastal Area Studies, School of Marine Sciences Alagappa University, Thondi Campus, Thondi – 623 409, Ramanathapuram District, Tamil Nadu, India; *Correspondence: vgs.biot@gmail.com / kshivam69@gmail.com; Tel: +91 9786330511

Abstract: The synthesis of nanoparticles has become a matter of great interest in recent times due to their various advantageous properties and applications in a variety of fields. We have reported biological synthesis of nano-sized silver particles. The nanoparticles of silver were formed by the reduction of silver nitrate to aqueous silver metal ions while effect of marine seaweed *U. reticulata* extract was investigated; silver nanoparticles were characterized using UV-visible absorption and room temperature photoluminescence. The X-ray diffraction results revealed that the synthesized silver nanoparticles were in the cubic phase. The existence of functional groups was identified using Fourier transform infrared spectroscopy. The morphology and size of the synthesized particles were studied with atomic force microscope. Further, the photocatalytic degradation of methyl orange was measured spectrophotometrically by using silver as nanocatalyst under visible light illumination. Silver nanoparticles synthesized from *U. reticulata* by facile method from can able to degrade dyes in the presence of visible light and paves way for ecological health and environmental bioremediation. Despite numerous studies conducted over the last decade, there are still considerable gaps in our knowledge about the biotechnological potential of green-synthesized nanoparticles. Furthermore, the precise basis of their antibiotic activity has yet to be defined. The biological methods are generally cost effective, nontoxic, and ecofriendly. This chapter focuses on the methods involved in algal-synthesized nanoparticles and its applications.

Keywords: AFM; Ag nanoparticles; antibacterial activity; cubic phase; methyl orange; photocatalytic degradation; *Ulva reticulata*

1. Introduction

Nanotechnology deals with the synthesis of nanoparticles that exhibit completely new or improved properties based on specific characteristics such as size, distribution and morphology. New applications of nanoparticles and nanomaterials are emerging rapidly (Jahn 1999; Naiwa 2000; Murphy 2008). Currently, there is a growing need to develop eco- friendly and sustainable

methods for the synthesis of nanomaterials that do not use toxic chemicals in the synthesis protocols so as to avoid adverse effects in medical applications. Among the nanoparticles, silver nanoparticles (AgNPs) have received considerable attention due to their attractive physico-chemical properties (Elechiguerra *et al.*, 2005). The metallic nanoparticles are most promising and considered as remarkable biomedical agents. Due to their large surface volume ratio, they govern

interest of researchers on microbial resistance. Among the developed nanoparticles, silver (Ag) nanoparticles are pertaining to have a wide range of application in the fields of physical, chemical and biological sciences. In the past decade, several kinds of the biological organisms (microbes, plants and seaweeds) have been employed and well-studied for the ability of silver nanoparticles (Ag-NPs) synthesis (Ramanathan *et al.*, 2011; Ahmad *et al.*, 2003; Shankar *et al.*, 2003; Mohanpuria *et al.*, 2008; Kumar *et al.*, 2012a). Application of green chemistry in synthesizing nanomaterials has vital role in medicinal and all technological aspects (Mondal *et al.*, 2011, Begum *et al.*, 2009). Biologically synthesized Ag-NPs have wide range of applications because of their remarkable physical and chemical properties. The literature on the extra cellular biosynthesis of Ag-NPs using plants and pure compounds from plants are insignificant (Kattumuri *et al.*, 2007; Song and Kim 2008; Gilaki 2010).

In this article, we describe a simple one step method for the synthesis of Ag-NPs by the reduction of aqueous Ag-ions using extracts of green seaweed, at direct sunlight conditions.

2. Materials and methods

2.1. Screening and selection of sample

Fresh sample of *Ulva reticulata* green seaweed was collected in the month of January 2013 from Pudumadam coastal region (78.99°E, 9.27°N), in Gulf of Mannar, Tamil Nadu, India. Sample was immediately brought to the laboratory in polythene bags and cleaned thoroughly with fresh water to remove adhering debris and associated biota. The alga sample was cleaned with distilled water using brush for the removal of the epiphytes.

2.2. Preparation of aqueous extract

The whole *U. reticulata* samples were initially rinsed thrice in distilled water and dried on paper toweling. Twenty five (25) gram sample was cut into fine

pieces and boiled in 100 ml of sterile distilled water for 5 min. The crude extract was passed through Whatman No.1 filter paper and the filtrate was stored at 4°C for further use the methods suggested by Jha *et al.* (2009).

2.3. Synthesis of silver nanoparticles

Analytical grade (AR) Silver nitrate (AgNO₃) was purchased from E. Merck (India). In the typical synthesis of silver nanoparticles, 10 ml of the aqueous extract of *Ulva reticulata* was added to 90 ml of 1 mM aqueous AgNO₃ solution in 250 ml conical flask and incubated at room temperature for 72 h by agitating at 120 rpm. Suitable controls were maintained throughout the experiments (Parashar *et al.*, 2009). The bio-reduction of AgNO₃ into AgNPs can be confirmed visually by the change in colour from light yellow to brown indicating the formation of silver nanoparticles (Figure 1).

2.4. Characterization techniques

The colour change in reaction mixture (metal ion solution + seaweed extract) was recorded through visual observation. The bio reduction of silver ions in aqueous solution was monitored by periodic sampling of aliquots (0.5 ml) and subsequently measuring UV-Vis spectra of the solution. UV-vis spectra of these aliquots were monitored as a function of time of reaction on UV-Vis spectrophotometer UV-2450 (Shimadzu).

The Ag-NPs solution thus obtained was purified by repeated centrifugation at 5000 rpm for 20 min followed by resuspension of the pellet of Ag-NPs in 10 ml of deionized water. After freeze drying of the purified Ag-NPs, its structure and composition was analyzed by XRD. The dried mixture of Ag-NPs was collected for the determination of the formation of Ag-NPs by an X'Pert Pro x-ray diffractometer (PAN analytical BV, The Netherlands) operated at a voltage of 40 kV and a current of 30 mA with Cu K α radiation in θ - 2θ configurations. The crystallite domain size was calculated



Figure 1: Silver nitrate (AgNO_3) solution and others colour changes during the reduction of AgNO_3 into AgNPs by the extract of *U. reticulata* after 20 min of incubation.

from the width of the XRD peaks, assuming that they are free from non-uniform strains, using the Scherrer's formula.

$$D = 0.94 \lambda / \beta \cos \theta$$

where D is the average crystallite domain size perpendicular to the reflecting planes, λ is the X-ray wavelength, β is the full width at half maximum (FWHM), and θ is the diffraction angle. To eliminate additional instrumental broadening the FWHM was corrected, using the FWHM from a large grained Si sample.

$$\beta_{\text{corrected}} = (\text{FWHM}^2_{\text{sample}} - \text{FWHM}^2_{\text{Si}})^{1/2}$$

This modified formula is valid only when the crystallite size is smaller than 100 nm.

The silver nanoparticles were observed using SEM. Sample was prepared by placing a drop of AgNPs on carbon coated copper stuff and subsequently drying air, before transferring it to the microscope operated at an accelerated and voltage of 120KV (JOEL Model JSM-5010 LV with INSA EDS) and followed for Energy Dispersive Spectrophotometer analysis.

The morphology of the product was observed by Nano Surf Easy Scan 2 Atomic Force Microscope (AFM) measurement to study the morphology and size of the Ag-NPs (Al-Warthan et al., 2010).

2.5. Photocatalytic degradation

The photocatalytic degradation of methyl orange was evaluated by biosyn-

thesized Ag-NPs (Rashed and El-Amin 2007). All the experiments were performed outdoor with sun as the main source of light (Wang et al., 2000). Prior to the experiment, a suspension was prepared by adding 20 mg of Ag-NPs to 50 ml of methyl orange solution (Fisher Scientific). Later, the mixture was allowed to stir constantly for about 30 min in darkness to ensure constant equilibrium of Ag-NPs in the organic solution. During the reaction, the mixture was kept under sunlight within a Pyrex glass beaker and stirred constantly. The mean temperature was found to be 29°C with 10 h mean shine duration. The absorption spectrum of the suspension mixture was measured periodically using a UV-visible spectrophotometer (Shimadzu, UV-2450, Japan) after centrifugation to ensure the degradation of methyl orange solution.

2.6. Antibacterial activities

Experimental pathogens namely, Gram positive bacterium *Staphylococcus aureus*, Gram negative bacteria *Pseudomonas aeruginosa*, *Escherichia coli*, *Proteus mirabilis* and *Proteus vulgaris* were obtained from Raja Sir Muthaiya Medical College, Annamalai University. Pathogens were loaded in inoculated in medium (Muller Hinton Agar medium) [100 µg/ml, 50 µg/ml, 25 µg/ml (Ag-NPs)] and AgNO_3 was used as negative control. Each culture was spread on to Muller Hinton Agar plates. Sterile paper discs of

6mm diameter along with streptomycin (Positive control) antibiotic containing discs were placed in each plate. Bacterial growth inhibition was determined as the diameter of the inhibition zones around the discs. All tests were performed in triplicate. Then, Petri dishes were incubated at 37°C for 18–24 h aerobically, inhibition zone were measured and data was recorded (Bauer *et al.*, 1966).

3. Results and discussion

3.1. UV–Visible spectroscopy

UV–visible absorption is one among the most important techniques to identify the formation of metal nanoparticles, provided surface plasmon resonance exists for metal (Binupriya *et al.*, 2010). It is well known that silver nanoparticles exhibit yellowish brown color in aqueous solution due to excitation of surface plasmon vibrations in silver nanoparticles (Shankar *et al.*, 2004). As the extract was mixed in the aqueous solution of the silver ion complex, it started to change the color from watery to yellowish brown due to reduction of silver ion which indicated formation of silver nanoparticles. It is generally recognized that UV–Vis spectroscopy could be used to examine size and shape controlled nanoparticles in aqueous suspensions (Wiley *et al.*, 2006). The UV–Vis spectra recorded from the reaction medium after 4 hours is shown in Figure 2. A strong silver plasmon absorption maximum was recorded at 410–420 nm in UV–Vis spectroscopy. The observed band in this range has been associated with Ag-NPs confirming the synthesis of spherical Ag-NPs with narrow size distribution has been revealed (Henglein 1993 and similar observation were also made by Kumar *et al.*, 2012b). Elevation in temperature results in formation of spherical and octahedral shaped nanoparticles of size 5–100 nm (Lengke *et al.*, 2007). Similarly, shape-controlled Ag-NPs can be also synthesized using biological system under varying temperature (Bansal *et al.*, 2012).

3.2. FTIR spectrum

FTIR analysis was used for the characterization of the extract and the resulting nanoparticles. FT-IR measurements were carried out to identify the possible biomolecules responsible for the reduction of Ag⁺ ions and the capping of the bio-reduced AgNPs synthesized. FT-IR spectrum (Figure 3) showed different major peaks positioned at 3405.37, 2955.75, 2922.87, 2851.82, 1654.15, 1637.15, 1512.61, 1457.45, 1418.38, 132.90, 1250.54, 1175.74, 1109.93, 812.05 and 699.73 cm⁻¹. The presence of peak at 3405.37 cm⁻¹ could be due to O-H group in alcohols and phenols. A small peak observed at 2955.75, 2922.87, and 2851.82 cm⁻¹ is due to C-H stretching of alkanes. Sharp and intense bands observed at 1654.15, 1637.15, 1560.38, 1512.61, and 1490.30 cm⁻¹ are due to –C=C– stretch, N-H bend, NO₂ asymmetrical stretch and nitro compounds, respectively. Another bands were positioned at 1457.45 (C-H bend alkanes), 1418.32 (C-C stretch (in-ring) aromatics), 1362.90 (C-H rock alkanes) and 1250.74 (C-N stretch aromatic compounds). The observed bands ranging between 1109.93 and 1032.31 cm⁻¹ are due to C-N stretch band of aliphatic amines. Bands observed at 812.05 and 743.34 cm⁻¹ are due to N-H wag band of primary and secondary amines. A band positioned at 699.73 cm⁻¹ is due to –C (triple bond) C-H; C-H bond alkynes. After bio-reduction, there is a shift in the absorption and band at 2955.75, 1457.45, 1362.90, 812.05 and 743.34 cm⁻¹ may be due to the binding of (NH) C=H and N-H wag group with the nanoparticles. The (NH) C=H groups within the cage of cyclic peptides are involved in stabilizing the nanoparticles. Thus, the peptides may play an important role in the reduction of Ag-NPs. This could be due to the ability of reducing and capping agents present in *U. reticulata* which were revealed by FT-IR studies. Fourier Transform Infra-Red (FT-IR) spectroscopy analysis showed that the

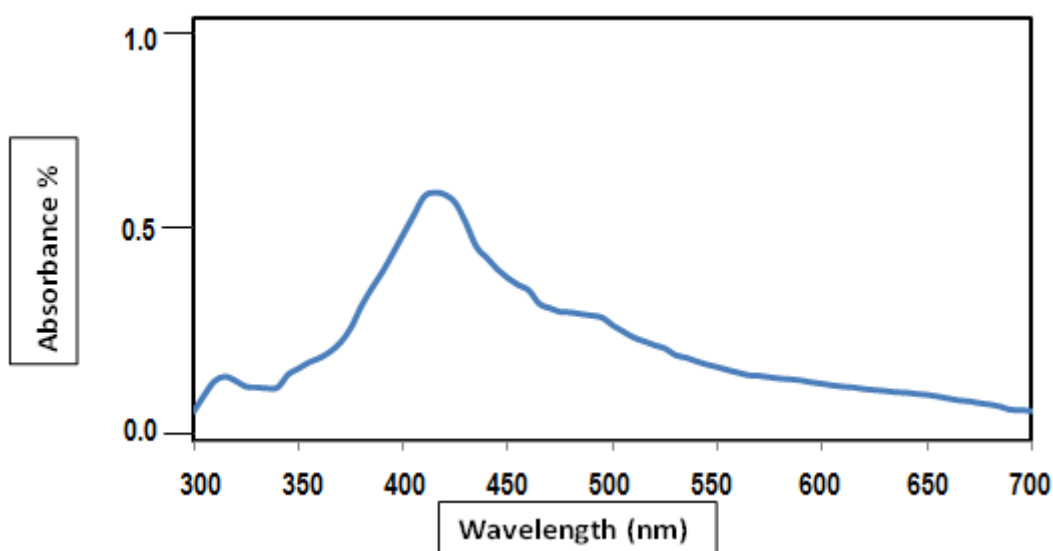


Figure 2: UV-visible absorption spectra of silver nanoparticles after 30 min of incubation.

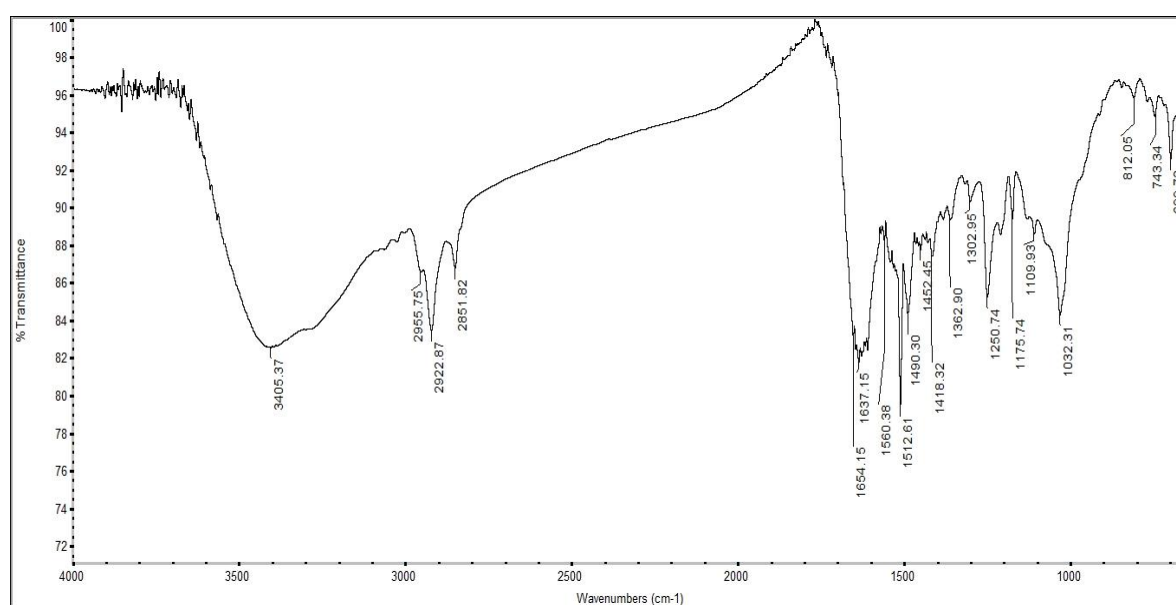


Figure 3: FT-IR spectra of AgNPs synthesized by *U. reticulata*.

synthesized nano-Ag was capped with bimolecular compounds which are responsible for reduction of silver ions (Jegadeeswaran *et al.*, 2012). The above-mentioned shift was also observed in *Codium capitatum* (Kannan *et al.*, 2013).

3.3. Crystal structures analysis and determination of crystallite size

The XRD pattern showed three intense peaks in the whole spectrum of 2θ value ranging from 10 to 80. Average size of the synthesized particles was 10 nm with size range 10 - 50nm with cubic and hexagonal shape. The typical XRD pat-

tern revealed that the sample contains a mixed phase (cubic and hexagonal) structures of silver nanoparticles. The average estimated particle size of this sample was 10 nm derived from the FWHM of peak corresponding to 90 plane (Figure 4). X-ray diffraction showed the average particle size of 15 nm as well as revealed their cubic structure (Geethalakshmi and Sarada 2010).

3.4. Particles morphology (SEM and AFM measurements)

The SEM image (Figure 5 a and b) depicts the high density Ag-NPs synt-

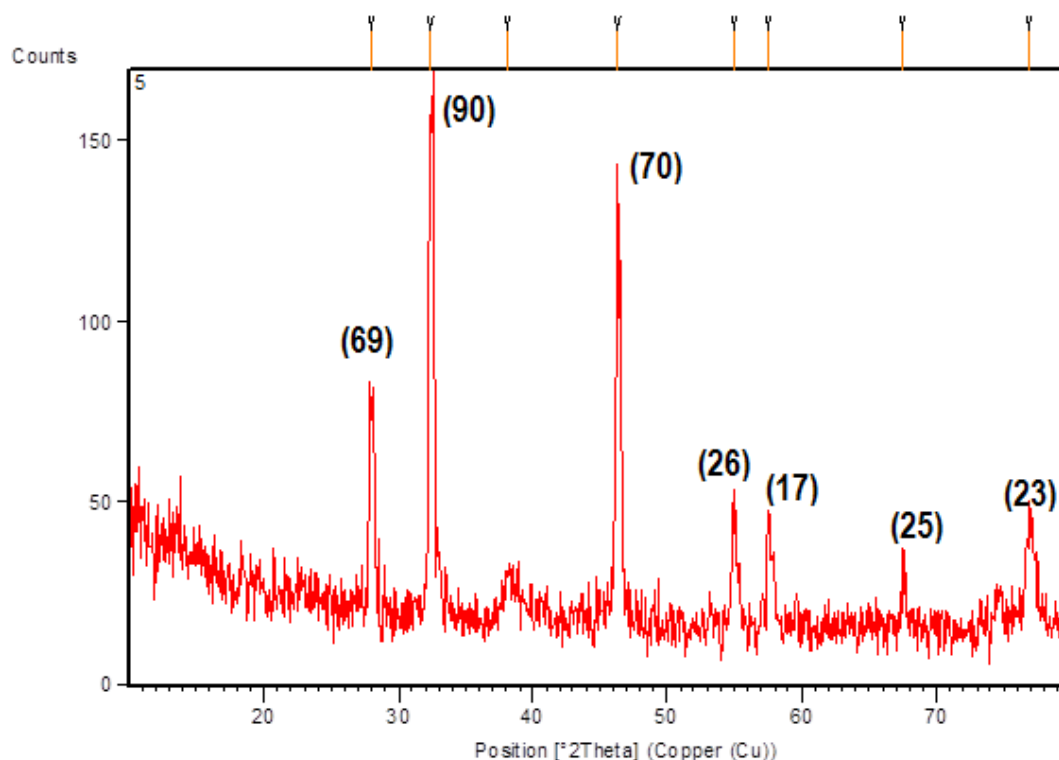


Figure 4: XRD patterns of silver nanoparticles synthesized after 120 h of incubation.

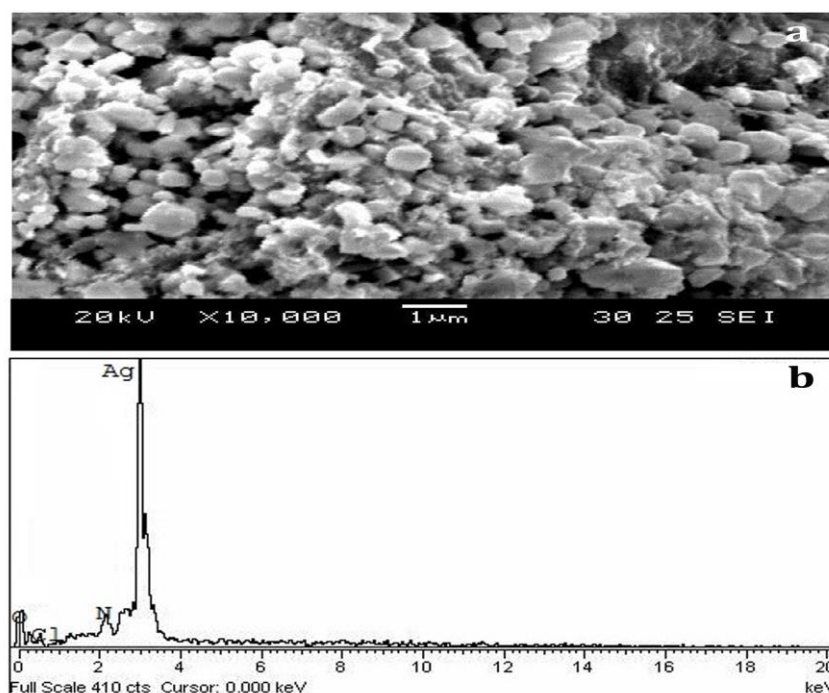


Figure 5: (a) SEM micrograph of Silver nanoparticles synthesized from the extracts of *U. reticulata*; (b) energy dispersive spectrometer analysis

-hesized by the *U. reticulata* and confirms the development of silver nanostructures with energy dispersive spectrometer. The SEM micrographs of nanoparticle obtained in the filtrate showed that Ag-NPs

are spherical shaped and well distributed without aggregation in solution. Ag-NPs predominantly spherical well distributed with an average size 15 nm (Saraniya Devi et al., 2013). It is known that the shape

of metal nanoparticles considerably changes their optical electronics properties (Xu and Kall, 2002). Similar phenomenon was reported by Chandran *et al.* (2006).

The surface morphology and size of the Ag-NPs harvested after 120 h of incubation were studied by AFM. The two- and three-dimensional images of the nanoparticles are shown in Figure 6a and 6b. From the 2D view, well-separated spherical particles are seen. The sizes of the particles are in the range of 1–40 nm. However, most of the particles are in the range of 10 nm. The 3D view revealed that the growth direction of all the particles was almost same confirming the single crystalline nature of the cubic phase of Ag-NPs. Williams, (2008) reported that nanoparticles are clusters of atoms in the size range of 1–100 nm. Morphology and size of the synthesized particles were studied with atomic force microscope (shanmugam *et al.*, 2014).

3.5. Photocatalytic degradation

Photocatalytic degradation of methyl orange dye was investigated using biometrically synthesized silver nanocatalysts by solar irradiation technique at different time intervals as shown in Figure 7. The characteristic absorption peak of methyl orange solution was found to be 420 nm. Degradation of methyl orange was visualized by decrease in peak intensity

within 10 h of incubation time. There is no considerable shift in peak position for methyl orange solution without exposure to Ag-NPs. Kansal *et al.* (2006) have reported that compared to other irradiation techniques, solar light was found to be faster in decolorizing methyl orange in the presence of metal catalyst. The adsorption of Ag-NPs on to the methyl orange solution was initially low and further increased with constant increase in time. Altogether, the photocatalytic properties of Ag-NPs in visible light may be well due to excitation of SPR, which is nothing but oscillation of charge density that can propagate at the interface between metal and dielectric medium (Garcia, 2012). Ag-NPs are potential, highly efficient and stable photocatalysts under ambient temperature with visible light illumination for degrading organic compounds and dyes (Wang *et al.*, 2008).

3.6. Antibacterial activities

Highest inhibition zone (10mm) in *Proteus mirabilis* was observed in *U. reticulata* at 100 µg/ml, lowest inhibition zone (7 mm) was observed in *U. reticulata* 25 µg/ml. *U. reticulata* at 100 µg/ml exhibits high inhibition zone of 8 mm in *Escherichia coli* and lowest inhibition zone (7 mm) was present in *U. reticulata* at 25 µg/ml. Ag-NPs from *U. reticulata* was compared effectively with silver nitrate solution and standard antibiotic

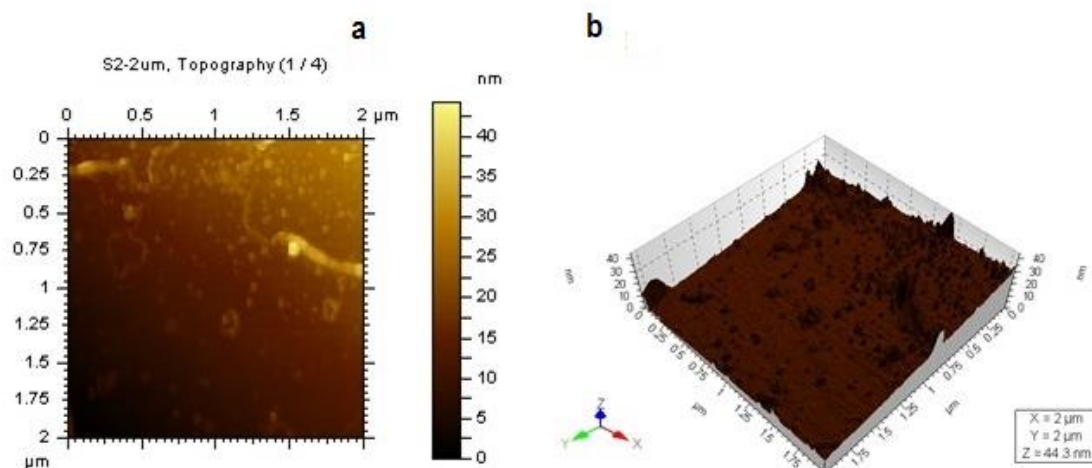


Figure 6: (a). AFM images of synthesized silver nanoparticles using extract of *U. reticulata*; (b) corresponding 3D view.

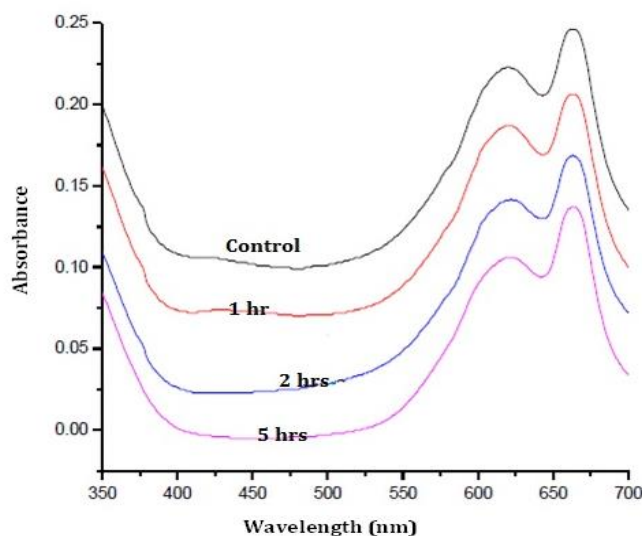


Figure 7: Photocatalytic degradation of methyl orange using silver nanoparticles synthesized from *Ulva reticulata*.

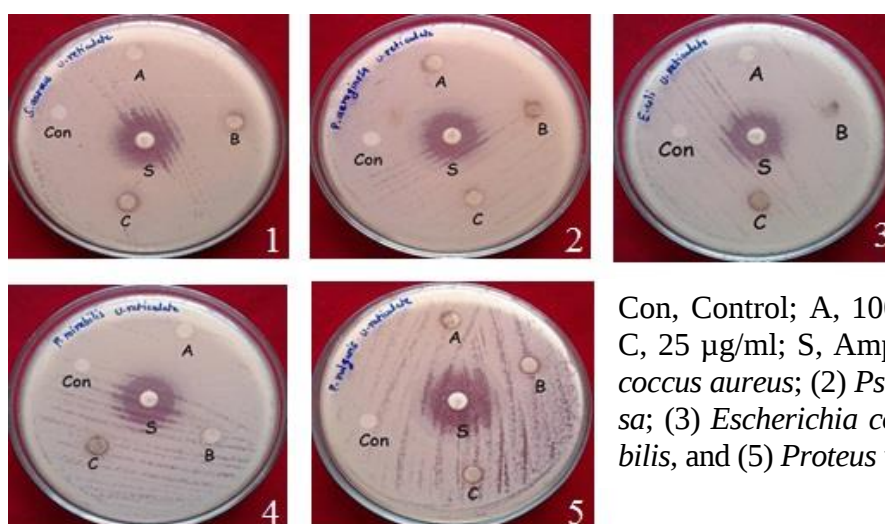


Figure 8: Antibacterial activity of silver nanoparticles synthesized from *U. reticulata* against human pathogens.

Con, Control; A, 100µg/ml; B, 50µg/ml; C, 25 µg/ml; S, Ampicillin; (1) *Staphylococcus aureus*; (2) *Pseudomonas aeruginosa*; (3) *Escherichia coli*; (4) *Proteus mirabilis*, and (5) *Proteus vulgaris*.

streptomycin, Ag-NPs exhibited more activity than silver nitrate solution. Maximum inhibitory activity was observed in Ag-NPs from *U. reticulata*, when compared to control. Raimondi *et al.* (2005) and Morones *et al.* (2005) corroborated that the bactericidal effect of silver nanoparticles is size dependent, the antimicrobial efficacy of the nanoparticle depend on the shapes of the nanoparticles also, this can be confirmed by studying the inhibition of bacterial growth by differentially shaped nanoparticles.

4. Conclusion

In this present investigation, the environmental friendly synthesis of Ag-NPs using fresh extract of the green seaweed *U. reticulata* is described. Despite numerous studies conducted over the last decade, there are still considerable gaps in our knowledge about the biotechnological potential of green-synthesized nanoparticles. Furthermore, the precise basis of their antibiotic activity has yet to be defined. In addition, improvements in the way that green-synthesized nanoparticles are incorporated into medical devices could increase their efficacy and diminish any side effects; but, further research is required to perfect this technology. The

morphology of silver nanoparticles was characterized by SEM and AM. The nanoparticles were found to be active in degrading methyl orange solution with visible light illumination. The antimicrobial activity of synthesized Ag-NPs is promising. In a nutshell, synthesis and characterization of Ag-NPs with regard to novel morphology are of great interest in the fabrication of antibacterial materials.

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Diversity and Ethno-Botanical Potential of Tree Plants of Katarniaghat Wildlife Sanctuary, Bahraich (UP) India: An Overview

Tej Pratap Mall*

Postgraduate Department of Botany, Kisan PG College, Bahraich-271 801, Uttar Pradesh, India; *Correspondence: drtejpratapmall@gmail.com; Te: +91 945 042 5622

Abstract: Plants are of variable purpose having efficiency as ethno-botanical, ethno-medicinal, ethno-veterinary and even in agro-forestry wherein they act as shelter, source of habitat for several organisms, etc. They even have a role in improving the soil conditions. Many useful products such as fruits, timber, fire wood and variety of metabolic chemicals are also obtained from plants. In *Katarniaghat Wildlife Sanctuary (KWS)*, there are fifty five tree plant species representing forty five genera belonging to thirty one families. *Moraceae* was found to be the largest family with seven plant species; whereas *Euphorbiaceae* and *Mimosaceae* with five; *Anacardiaceae*, *Myrtaceae* and *Rubiaceae* with three; *Caesalpiniaceae*, *Ehretiaceae*, *Papilionaceae* and *Louraceae* with two and rest twenty one families, viz., *Rutaceae*, *Apocyanaceae*, *Baringtoniaceae*, *Bombocaceae*, *Dilleniaceae*, *Ebenaceae*, *Tiliaceae*, *Ulmaceae*, *Malvaceae*, *Lythraceae*, *Sapotaceae*, *Annonaceae*, *Rutaceae*, *Sapindaceae*, *Dipterocarpaceae*, *Sterculaceae*, *Bignoniaceae*, *Verbinaceae*, *Combretaceae*, *Meliaceae* and *Rhamnaceae* with single plant species only. This chapter is an attempt to summarise the information available on plant species found in KWS which are yet not popular due to limited research.

Keywords: Ethnobotanical; ethnomedicinal; ethnoveterinary; Katarniaghat wildlife sanctuary; nutrimental tree

1. Introduction

We cannot survive without plants. We depend on plants for food: directly in the form of grains, roots and tubers, fruits, vegetables, spices, oil and beverages. Much of our food also comes indirectly from plants. We get our meat and milk from animals that are dependent on plants for food. Plants provide fuel, either as firewood or in the form of fossil fuel, to cook our food, keep us warm, run our machinery and light up our homes and cities. We also depend on trees for construction materials to build our houses and to craft our furniture. From cotton and flax we get fibres for our clothes. Plant dyes colour our clothes, at least before synthetic dyes were developed. In

cities and towns, trees provide shade and shelter, and their flowers brighten the surroundings. Plants in parks and gardens contribute to the serene and peaceful environment, making such places favourite retreats (Chin, 2005).

The knowledge of utilizing wild plants was painstakingly passed on from generation to generation database of valuable information of the plants around him. It is natural to assume that certain members of the tribe were gradually entrusted with such knowledge. These individuals were known as shamans, bomohs, healers or witchdoctors. As communications between settlements was then poor, it is likely that such knowledge was developed independently in different locations (Chin, 2005). The primitive

man, through his trial and error, has selected many wild fruits which are edible and subsequently domesticated them which played a very vital part in supplementary diet knowingly or unknowingly. Although due to the ignorance of modern generation the importance of wild plants were recently have been decreasing yet many people especially in rural areas still use them extensively as a supplementary to their basic food requirement. A scientific study of wild fruits is important for the potential sources which are protective foods. The nutrients/pigments present in the fruits prevent different degradative/ageing process in our body and thus via restoring health offer longevity (Singh, 2011). These wild fruits would be utilized at the time of scarcity or cultivated as a source of food material for ever increasing population (Rashid *et al.*, 2008).

India has been considered as one of the 17 mega-diversity centers of the world with a wide range of phyto-geographical variations. It consists of about 64 million hectares forest covers out of which 86% is tropical forest comprising 54% dry deciduous, 37% moist deciduous and 9% wet evergreen & semi-evergreen (Kaul and Sharma, 1971). As a characteristic feature, the tropical forest shows a huge variation in tree species diversity place to place (Pitman *et al.*, 2002). Among the different phyto diverse regions found in the country, the Terai region is one of them existing from Uttarakhand to West Bengal. It is the transition zone between two eco-climatic zones, the Gangetic plain towards south and Bhabhar towards north, along with the sub- Himalayan tracts (Tripathi and Singh, 2009). The region has lost majority of its natural forest due to deforestation chiefly for agriculture and lack of sustainable forest management in last many centuries (Bajpai *et al.*, 2012a, b). Now the natural forests of the region have been restricted to the wildlife protected areas only. *Katerniaghat* Wildlife Sanctuary (KWS) is also one of them.

Traditional medicines are used by about 60 percent of the world's population. These are not only used for primary health care just in rural areas, in developing coun-

tries, but also in developed countries, where modern medicines are predominantly used. While the traditional medicines are derived from medicinal plants, minerals, and organic matter, the herbal drugs are prepared from medicinal plants only. Use of plants as a source of medicines has been inherited and is an important component of the health care system in India. There are about 45,000 plant species in India, with high concentration in the region of Eastern Himalayas, Western Ghats and Andaman and Nicobar Island. The officially documented plants with medicinal potential are 3,000 but traditional practitioners use more than 6,000. India is the largest producer of medicinal herbs and is appropriately called the botanical garden of the world. In rural India, 70 percent of the population is dependent on the traditional system of medicine, the Ayurveda, which is the ancient Indian therapeutic measure renowned as one of the major systems of the alternative and complementary medicine (Bhatia, *et al.*, 2013).

In *Katerniaghat* Wildlife Sanctuary there are fifty five tree plant species representing forty five genera belonging to thirty one families. *Moraceae* was found to be the largest family with seven plant species whereas *Euphorbiaceae* and *Mimosaceae* with five; *Anacardiaceae*, *Myrtaceae* and *Rubiaceae* with three; *Caesalpiniaceae*, *Ehretiaceae*, *Papilionaceae* and *Louraceae* with two and rest twenty one families, viz., *Rutaceae*, *Apocyanaceae*, *Baringtoniaceae*, *Bombocaceae*, *Dilleniaceae*, *Ebenaceae*, *Tiliaceae*, *Ulmaceae*, *Malvaceae*, *Lythraceae*, *Sapotaceae*, *Annonaceae*, *Rutaceae*, *Sapindaceae*, *Dipterocarpaceae*, *Sterculiaceae*, *Bignoniaceae*, *Verbinaceae*, *Combretaceae*, *Meliaceae* and *Rhamnaceae* with single plant species only. The available literature reveals that most of the tree plants found in *Katerniaghat* Wildlife Sanctuary (KWS) are multi-purpose, ethno-botanical, nutrimental, ethno-medicinal, ethno-veterinary and of environmental use in agro-forestry which provide shade, habitat for organisms, soil improvement, etc., many useful products are also obtained such as fruits, timber, fire wood and variety of metabolic chemicals which may be used in the form of home remedies and for traditional medicine. Considering the multi-purpose importance of these trees of KWS, the present overview is an attempt to summarize the information's available on these plants which are yet not popular due to one

reason or the other despite providing an array of benefits.

2. Study area

The study area *Katerniaghat* Wildlife Sanctuary (KWS) is situated in Bahraich district of Uttar Pradesh in India. It lies along Indo-Nepal international border and is situated between 27° 41' – 27° 56' N and 81° 48' – 81° 56' E covering an area of 440 km² with 116 to 165 m elevation. The sanctuary comes under the tropical moist deciduous forest of the Himalayan Terai-Bhabar region (Champion and Seth, 1968; Rodgers and Panwar, 1988). The forest of the sanctuary area has been classified into two major forest types (i) The Sal forest and (ii) The miscellaneous forest (Champion and Seth, 1968). Pedagogically the study area is made up of the alluvial soil of the *Kaudiyala* and *Saryu* rivers and its tributaries flowing adjoining to it. Geologically the sanctuary area has been divided into high and low land areas.

3. Climate

A typical tropical monsoonal climate with three distinct seasons, i.e., summer (April to June), winter (November to February) and warm-rainy (July to September) prevails in the study area. March and October are considered as transition months between the seasons. The mean maximum temperature ranges from 22°C in January to 40°C in May and the mean minimum temperature ranges from 8°C in January to 27°C in June. The annual rainfall ranges from 36 to 142 cm in winter, 34 to 662 cm in summer and 1294 to 1689 cm in warm-rainy seasons (Bajpai *et al.*, 2012).

4. Observations

At present the KWS has been divided in to three types of forests- miscellaneous forest, sal forest and teak plantation forest. The IVI of the plants in all the three forests are presented respectively

while description of the plant. Since the list of the trees is big, the description of all is beyond the scope of this manuscript so we have taken thirteen plants, viz., *Acacia catechu*, *Acacia concinna*, *Aegle marmelos*, *Albizia lebbek*, *Albizia procera*, *Alstonia scholaris*, *Bombax ceiba*, *Diospyros cordifolia*, *Ficus racemosa*, *Madhuca latifolia*, *Shleichera oleosa*, *Syzygium cumuni* and *Ziziphus mauritiana* for detail description.

4.1. *Acacia catechu* Willd. Khair, Catechu (Mimosaceae)

It is a moderate size tree. Leaves are pinnate. Flowers yellow in globose, peduncled axillary heads. Pods strap shaped, dark brown. Phenology - August to February. In *Katerniaghat* Wildlife Sanctuary it is found only in miscellaneous forest with IVI value 18.3.

Ethnobotanical potential

- Catechu, a multipurpose tree species is widely used by the inhabitants for fodder, fuel, building material and in health care.
- The heartwood of the tree is mainly used for extracting *Katha* and Cutch (decoction obtained after filtration) which are sold in the market.
- *Katha* is commonly used in ayurvedic preparations.
- *Katha* serves as one of the major components in masticatory, i.e., chewing of betel leaf (pan) in India.
- *catechu* is a valuable bio-resources and has been exploited commercially in tannin and *Katha* industry for decades.
- Besides its commercial importance, it is equally significant for the people particularly rural communities living in the vicinity of catechu forests as it is a subsidiary source of income to them. To a certain extent, these people are dependent on this plant to fulfill their day to day need of fuel, fodder, building material, *etc.*

Ethno-medicinal potential

- The decoction of bark mixed with milk is taken to cure cold and cough.
- The bark decoction is either alone or used in combination with opium to cure severe diarrhea.
- *Katha* after drying is applied on lemon slice and taken regularly with empty stomach to cure piles.
- Heartwood of *khair* is boiled with other ingredients to prepare the decoction. It is taken as tea by the pregnant ladies to keep warm their body. It is also given to cure fever due to cold during the pregnancy.
- A decoction is served to women after 2-3 days of child delivery, prepared by boiling *Katha* along with cardamom. It is believed that it provides strength to the body and also helps in secretion of milk.
- The water boiled with the heartwood chips of *Khair*, is used to take bath by women after delivery. It is considered beneficial to cure the body pains.
- *Katha* or decoction of heartwood is applied in mouth and on tongue to cure mouth ulcer. It is also applied externally on ulcers, boils, skin eruptions and on gums as disinfectant.

Fuel

- The dried logs, twigs and branches are largely used as fuel.

Fodder

- The trees are lopped heavily for their leaves used as fodder particularly for sheep and goats.

Building and furniture material

- The wood is considered durable and widely used by the inhabitants for house building material as pole and to prepare furniture like bed poles, tables etc.

House hold articles

- Wood of *khair* is preferred to prepare various parts of local plough,

handles of axe, saw, sickle, hammer, spade and combs.

Socio-religious beliefs

- *Khair* is considered one of the sacred trees by the natives and wood is used in the religious ceremonies at the time of havans (yagya).
- Wood is considered sacred and used as one of the religious plants along with *bhoj patra* (*Betula utilis*) at the funeral ceremony. It is believed to provide *mukti* or *moksha* (peace to the heavenly soul).

Fencing

- Cut branches are extensively used for fencing purpose by the farmers to protect agricultural fields and local grasslands from domestic livestock and wild animals.

Tanning

- The cutch is used locally for tanning leather and as dye to a great extent.

Economic Importance

- Besides traditional utility, *A. catechu* is widely utilized commercially for extracting *Katha* from the heart wood which costs around US \$ 4-6 per kilogram in Indian markets.
- Cutch is used as adhesive in plywood industry and it is also used in preparing polishes and paints (Singh and Lal, 2006).

From the present study, it is envisaged that *A. catechu* has a great socio-economic importance as it is widely used for different purposes by the natives. Besides, traditional and commercial importance, it has tremendous ecological significance. Because of its leguminous nature and soil binding abilities, it is a suitable species for wasteland development.

4.2. *Acacia concinna* (Willd.) DC

Synonyms: *Acacia sinuata* (Lour.) Merrill, *Acacia rugata* Lamk., *Mimosa sinuate* Lour.

Acacia concinna is a prickly bush or climbing shrub and grows in tropical forests of India, common in the warm plains leaflets membranous. The leaves are bipinnate and the thorns are soft and small. Flowers are white and pink in copiously paniced globose heads in the axils of leaf. Pods strap shaped, straight, thick, succulent when dry shriveled and rough with straight waved sutures. There are about 6-10 seeds in a pod. Phenology is January-March & November to February. The tree is food for the larvae of the butterfly *Pantoporia hordonia*. Alkaloids are found in the tree's fruit. In *Katarniaghat* Wildlife Sanctuary it is found only in miscellaneous forest with IVI value of 1.0

Ethno-botanical potential

- The leaves, pods has astringent action and useful in treating cuts, wounds and oral problems. The decoction of pods is prepared and used for washing and cleaning of wound for quick healing. *Acacia concinna* is a good herbal remedy for hair. It is an advantageous herb for baldness. Its usage helps in preserving the natural oil of our hair and nurtures the scalp. It encourages the growth of hair and strengthens them. It is an effective hair cleanser. It prevents dandruff and lice.
- It is used in the manufacture of shampoos, soaps and hair packs.
- It is a good herbal treatment for black fever (Visceral Leishmaniasis) and fever due to Malaria.
- *Acacia concinna* is a herbal treatment for skin ailments. It is advantageous in curing psoriasis (genetic disease) and the spreadable diseases like eczema. It provides a relief in scabies, rashes, cuts, bruises and cures them.
- It is effectual in curing oral ailments. It helps in suppressing bad breath. It is helpful in treating

mouth ulcers, gumboils and pain in the throat.

- It aids in the prevention of tooth degradation and formation of plaque.
- It is also helpful in lowering the chances of encountering diabetes.
- *Acacia concinna* is a good herbal treatment for lowering the body cholesterol.
- It is a fruitful remedy in curing digestive disorders and relieving constipation. It facilitates proper bowel movement and improves the flow of urine.
- It also possesses the attribute of being a contraceptive which helps in birth control.
- It is utilized in the preparation of savoury jams (chutneys). It adds to the flavor.
- *Acacia concinna* has been used traditionally for hair care in the Indian Subcontinent since ancient times.
- It is one of the Ayurvedic medicinal plants.
- Fruit for hair are being used as a traditional shampoo. In order to prepare it the fruit pods, leaves and bark of the plant are dried, ground into a powder then made into a paste. While this traditional shampoo does not produce the normal amount of lather which are found in sulfate-containing shampoo, it is considered a good cleanser. It is mild, having a naturally low pH, and doesn't strip hair of natural oils. Usually no conditioner is needed, for shikakai which also acts as a detangler.
- An infusion of the leaves has been used in anti-dandruff preparations.
- Since *A. concinna* extracts are used in natural shampoos or hair powders so the tree is now grown commercially in India.
- The plant parts used for the dry powder or the extract are the bark, leaves or pods. The bark contains high levels of saponins, which are-

foaming agents found in several other plant species used as shampoos or soaps. Saponin-containing plants have a long history of use as mild cleaning agents.

- Saponins from the plant's pods have been traditionally used as a detergent, and in Bengal for poisoning fish; they are documented to be potent marine toxins.
- The leaves have an acidic taste and are used in chutneys.

Chemical constituents

- In commercial extracts, when the plant is hydrolyzed it yields lupeol, spinasterol, acacic acid, lactone, and the natural sugars glucose, arabinose and rhamnose. It also contains hexacosanol, spinasterone, oxalic acid, tartaric acid, citric acid, succinic acid, ascorbic acid, and the alkaloid scalctomine and nicotine.
- *Acacia concinna* is a thorny medicinal plant, native to south Asia, widely known for the organic shampoo derived from its fruit, shikakai.
- The pods (shikakai), have medicinal properties and are used for hair cleansing and enhancement. Shikakai is traditionally preferred over commercially available shampoo, across the Indian Subcontinent.
- Shikakai is also used for manufacturing body and facial care creams, across the personal hygiene industry.
- A particular extract from *Acacia concinna* leaves has shown to be effective in treatment of malarial fever.

Ethno-medicinal potentiality

- Shikakai is also used in traditional medicine to treat jaundice, constipation and skin problems.

Acacia concinna for pain

- Apply some castor oil on the affected area. Heat with a hot water bag. Now massage with powdered

Acacia concinna for 15 minutes. Wash the area with hot water and wipe it.

Acacia concinna for constipation:

- Discard the *Acacia concinna* seeds after crushing the fruit. Soak it in 1 glass of water for one hour. Take the infusion.

Acacia concinna (shikakai) for jaundice

- Take out the *Acacia* seeds after crushing the fruit. Soak it in a glass of water for an hour. Take one fourth of the glass infusion.

Acacia concinna (shikakai) for dandruff

- *Acacia concinna* (shikakai) is a boon for getting rid of dandruff. Boil a handful of coarsely crushed *Acacia concinna* (Shikakai) in a litre of water for 10 minutes. Cool it and use the filtered decoction to wash hair. Do it daily for week and then twice a week or make a paste of powdered *Acacia concinna* (Shikakai) by adding water in it. Apply it over scalp and hair. Leave it for an hour. Wash hair with normal water. It also cleans your hair from roots to tips.

Acacia concinna for age spots

- Make a fine paste of *Acacia concinna* fruit. Apply every day for 15 minutes. Wash three times a day.

Acacia concinna for gum diseases

- Take half table spoon *Acacia concinna* and boil it in two cups of water. Gargle with this lukewarm water three times a day.

Acacia concinna for skin diseases

- Boil one table spoon *Acacia concinna* powder in one cup water. Cool it and apply on the skin.

Acacia concinna (shikakai) pods for leprosy

- Squeeze the juice of *Acacia concinna* (shikakai) pods. Apply it on effected parts twice a day.

Acacia concinna for psoriasis:

- Take few pods of *Acacia concinna*. Boil them. Apply them over affected areas.

Herbal treatment for constipation

- Make a paste of three leaf buds of *Acacia concinna*, two cloves of garlic and some black salt. Take it with cooked rice.

Herbal treatment for fever

- Make a paste of three leaf buds of *Acacia concinna*, two cloves of garlic and some common salt. Take it with cooked rice.

Herbal treatment for dandruff

- Grind three to four dried fruits of *Acacia concinna* (shikakai) without seeds. Add one to two teaspoons of fenugreek (methi in India) seeds, one teaspoon full wild turmeric (aamahaldi in India) root powder, one teaspoon of Indian Sarsaparilla (anantmool in India) root powder and one teaspoon of sandalwood powder. Mix well. Add water to form a thick paste. Massage the scalp with coconut oil, and then apply this paste. Leave it for half an hour. Wash off and shampoo. Do this once a week.
- Massage your scalp with lukewarm sesame oil. Boil four to five dried *Acacia concinna* (Shikakai in India) pods in two glass of water. Strain well. Let it cool. Use its water to rinse your scalp after an hour. It gives relief from dandruff and promotes hair growth.
- *Acacia concinna* (shikakai) and lemon both are beneficial herbs in treating dandruff. The mixture made up of these two, works wonder to make your hair free of

dandruff. Take 3 to 4 tablespoon of *Acacia concinna* (shikakai) powder. Make a paste by adding lemon (nimbu) juice. Apply it over scalp and leave it for an hour or two. Wash with water. Do it for a week every day and you may see half your dandruff has gone.

Herbal treatment for head lice

- Grind ten dried fruits of *Acacia concinna* after discarding the seeds, half cup each of fenugreek seeds, wild turmeric roots, roots of Indian Sarsaparilla and Sandalwood chips. Massage the head with coconut oil and apply this powder. Rinse off after thirty minutes.

Herbal treatment for jaundice

- Make a paste of one tea spoon tender *Acacia concinna* leaves, three pepper corns, one tea spoon tamarind pulp, half red chilli and some salt to add taste. Eat it with cooked rice.

Herbal treatment for stomach ache

- Boil one cup *Acacia concinna* tree bark in one litre water and sieve it. Add powdered Indian Pennywort in it. Also add the powder of four black peppercorns, two cardamom, cinnamon, two cloves, one fourth nutmeg and half tablespoon long pepper. Mix it and leave it for a month before it is taken for medicinal use. Take one tablespoon twice a day.

Herbal treatment for grey hair

- Soak Indian gooseberry, soap nut seeds (Ritha) and pods of *Acacia concinna* (Shikakai) in three cups of water for a night. Grind it. Use it as shampoo.

Herbal treatment for frizzy hair

- Take following herbs in mentioned quantity, two hundred g In-

dian gooseberry (*Amia*), two hundred g *Acacia concinna* (Shikakai), twenty g *Bacopa monnieri* (Brahmi), one g Asphaltum (Shilajit), forty g dried pomegranate peel (Anar ka chilka), twenty g *Eclipta alba* (Bhringraaj), Soak them overnight in any iron bowl. Grind to make paste. Apply it on your hair and Scalp. Wash after 3 hours with lukewarm water. Do not use shampoo. Shampoo may be used on next day.

4.3. *Aegle marmelos* (L.) Corr

It is a medium-sized, deciduous, armed tree. Leaves are trifoliate. Flowers are yellowish. Fruits are large, globose. Phenology is April-May & March-July. In *Katerniaghat Wildlife Sanctuary Aeglemarmelos* is found only in teak plantation forest.

Ethnobotanical potentiality

Bael is one of the most important tree species used in various indigenous system of medicine in India, China, Burma, and Sri Lanka. Bael is used in all *tridosha*- vista (air), *Pitta* (phlegm) and *kapha* (cough). Out of more than 66 ethno-botanical uses of bael, 48 are exclusively for medicinal purposes. Almost all parts of bael are used in preparing medicine (Kala, 2006).

- **Leaf:** Abscess, backache, eye complaints, abdominal disorders, vomiting, cut & wounds, ulcer, destroy, beriberi, weakness of heart, cholera, diarrhoea, cardio tonic, blood sugar, injuries caused by animals, nervous disorders, hair tonic, acute bronchitis, child birth, veterinary medicine for wounds, killing worms, fodder for sheep, goat and cattle, stimulation of respiration and contraction of de-nerved nictitating membrane in anaesthetized cats.
- **Fruit:** Astringent, diarrhoea, gastric troubles, constipation, laxative, tonic, digestive, stomachic, dysentery, brain

& heart tonic, ulcer, antiviral, intestinal parasites, gonorrhoea, epilepsy.

- **Root:** Dog bite, gastric troubles, heart disorders, intermittent fevers, antamoebic, hypoglycemic, rheumatism.
- **Bark:** Stomach disorders, intermittent fevers, heart disorders.
- **Seed:** Febrifuge.
- **Flower:** Expectorant, epilepsy.
- **Whole Plant:** Abdominal pain, abscess, astringent back ache, dog bite, breast pain, cholera constipation, convulsions, cramp, diabetes, diarrhoea, dysentery, fevers, eye complaints, gastric trouble, abdominal disorders, jaundice, laxative, nausea night fever, heart disorders, snakebite, stomach disorder, vomiting tonic, cut & wounds.
- **Root bark:** Fish poison.
- **Seed mucilage:** Plaster for walls.
- **Seed oil:** Laxative.
- **Wood:** Beads worn by low caste, special couches for rheumatic patients.
- **Gum around seed:** To improves adhesive strength of water paints.
- **Unripe fruit rind, Bark:** Yellow dye.
- **Stem:** Pestles of oil and sugar mills. The medicine is prepared in the form of pills, powder and paste. Ayurvedic practitioners commonly use the roots of bael as an ingredient of dasmula (ten roots), which is useful in recovering the loss of appetite and use fruits in the preparation of chawanprash.
- Bael fruits regarded as an astringent are frequently used by various ethnic communities for the treatment of diarrhoea, dysentery, constipation, stomach ache, intestinal ulcer, diabetes, dyspepsia, heart diseases and cholera due to its digestive and carminative properties.
- Bael is highly valued in Ayurvedic medicine for the treatment of chronic diarrhoea and dysentery and as brain tonic. Bael possesses antiviral, anti-helminthic anti-inflammatory, anti-bilious, anti-parasitical, anti-pyretic, anti-scorbutic, aromatic, astringent, digestive, febrifuge, haemostatic, anti-

diarrheal, laxative and nutritive properties.

Ripe bael fruit is sweet, aromatic and nutritive, whereas fresh fruit is stringent and has laxative properties. Bael fruit powder exhibits anticancerous and anti-proliferative activities. The combinations of five parts of bael, such as, fruit, leaf, bark, root and flower is assumed to be effective for certain mental disorders.

- Unripe fruits pulp mixed with boiled rice water is taken twice a day to cure vomiting in pregnancy. Unripe fruits pulp mixed with sugar is taken with milk twice daily for curing urinogenital disorders.
- Half roasted unripe fruit pulp mixed with equal quantity of sugar is taken twice a day to cure dysentery. Unripe fruit pulp powder is taken twice daily to cure abscess.
- Bael leaf extract is taken twice a day to remove the intestinal worms. Leaf poultice is used as remedy in ophthalmic problems and ulcer.
- Leaf juice is reported to have multiple medicinal uses, including controls of diabetes. Cooling delicious drink prepared from fruit pulp along with sugar and tamarind diluted with water is useful for health.
- Baelroot decoction is given twice daily to cure fever and cold. Extract of bael root, pyaz (*Allium cepa* Linn.), and haldi (*Curcuma domestica* Valetton) mixed in equal proportion is put in the ears to relieve earache and secretion from ears. Root decoction is used in the treatment of intermittent fevers and heart palpitation.
- Root and stem bark decoction is used in the treatment for fever and various types of heart disorders. Bael root is used in the treatment of abdominal pain, heart palpitation and urinary troubles.
- Bael tea is good for health and is used for flatulence, gastrointestinal problems, cough and chronic intestinal diseases in children.

- For Hindus, the Bael is sacred tree, which they dedicate to the lord Shiva by offering of Bael leaves. Its three leaflets are assumed by the symbols of three gunas or attributes (e.g., satva, rajas and tamas, literally meaning morality, superiority and immorality, respectively); three Gods (Brahma, Vishnu and Mahesh); and three lives (past, present and future). Bael is considered to be extremely auspicious and cultivated around most of the Hindu temples.

Phytochemicals of Aegle marmelos

A. marmelos has been reported to contain several phyto-constituents mainly marmenol, marmin, marmelosin, marmelide, psoralen, alloimperatorin, rutaritin, scopoletin, aegelin, marmelin, fagarine, anhydromarmelin, limonene, a-phellandrene, betulinic acid, marmesin, impertorin, marmelosin, luvangentin and auroptene Rahman and Parvin .

- Due to the presence of various phyto-constituents the plant has anti-diarrhoeal, anti-microbial, anticancerous, anti-pyretic, anti-genotoxic, anti-fertility, anti-inflammatory anti-diabetic and diuretic activities.
- The essential oil isolated from the leaves of *A. marmelos* tree has proved to have antifungal activity against animal and human fungi like *Trichophyton mentagrophytes*, *Trichophyton rubrum*, *Microsporum gypseum*, *Microsporum audouinii*, *Microsporum cookie*, *Pidermophyton floccosum*, *Aspergillus niger*, *Aspergillus flavus* and *Histoplasma capsulatum*.
- The leaf extracts and fractions have fungicidal activity against various clinical isolates of dermatophytic fungi.
- Various extracts of *A. marmelos* leaves, roots and fruits have been reported to be active against many bacterial strains.

Ethno-botanical potential

- Bael fruits are edible, contain high protein and are used in making tasty aromatic cold drinks and jam. Its fresh juice is better and pungent. In Myanmar, Bael fruits are used in making paints. Fruits are also used as a substitute for soap, as source of essential oils and perfumes. The mucilage of bael seed is good cementing material. Bael wood is used in building houses, making carts, agricultural implements, pestles, handles of tools and combs. A yellow dye is obtained from the rind of unripe fruits and is used in calico printing. An essential oil is also distilled from the rind. Dried fruit after removing the pulp are used as pill boxes for keeping valuable medicines and sacred ashes. Bael stem yields gum, which is used for improving the adhesive potency of water paints. Its wood is suitable for making charcoal.

4.4. *Albizzia lebbeck* (Linn.) Benth.

Synonym: *Mimosa lebbeck* Linn.

- It is a tall, unarmed, and deciduous tree distributed throughout India from the plains up to 900m in the Himalayas. It is tree growing to height of 18-30 m tall with a trunk 50 cm to 1 m in diameter. The leaves are bipinnate, 7.5-15 cm long, with one to four pairs of pinnae, each pinna with 6-18 leaflets. The flowers are white, with numerous 2.5-3.8 cm long stamens, and very fragrant. The fruit is a pod 15-30 cm long and 2.5-5.0 cm broad, containing six to twelve seeds. In Katariniaghat Wildlife Sanctuary it is found only in miscellaneous forest with IVI 0.9.

Ethnobotanical potential

- It uses included in environmental management, forage, medicine and wood. It is cultivated as a shade tree in North and South America. In India and Pakistan, the tree is used to produce timber. Wood from *Albizia leb-*

beck has a density of 0.55-0.66g/cm³ or higher (Babu *et al.*, 2009).

- Even a where it is not native, some indigenous herbivores are liable to utilize lebbeck as food resource. For example, the greater rhea (*Rhea Americana*) has been observed feeding on it in the cerrado of Brazil.

Ethno-medicinal potential

- Lebbeck is an astringent, also used by some cultures to treat boils, cough, to treat the eue, flu, gingivitis, lung problems, pectoral problems, is used as a tonic, and is used to treat abdominal tumors.
- The bark is used medicinally to treat inflammation.
- In Sidha system of medicine the bark and flowers of this plant are used to treat arthritis (Mudaliar, 1936).
- The tribal people in Himachal Pradesh and Kashmir use this plant to treat inflammation (Srivastava *et al.*, 1986; Jain, 1991; Kapur, 1993).
- Balasubramaniam (1992) reported that the tribals point Calimere Wildlife Sanctuary, Tamilnadu use this plant to treat fractures.
- In Ayurvedic system of medicine, the stem bark of this plant is used to treat diarrhoea (Nadkarni, 1954), edema, poisoning, asthma and bronchitis (Gupta, 2004).
- Inflammation is complex pathophysiological process medicated by a variety of signalling molecules produced by leucocytes, macrophages and mast cells as well as by the activation of complement factors that bring about edema formation as a result of extravasation of fluid and proteins and accumulation of leucocytes at the inflammatory site (White, 1999). All the steroidal and non-steroidal anti-inflammatory drugs (NSAID's), despite their great number, cause undesired and serious side effects. Therefore, development of new and more powerful drugs is still needed.

- Research in plants with medicinal properties and identification of the chemical components responsible for their activities have corroborated the traditional uses of ancient healing wisdom and lore and have proven the enduring healing potential of many plant medicines even in today's hi-tech community.
- It is previously reported that the alcoholic extract of *Albizia lebback* protects the guinea pig against the antigen induced challenge (Tripathi *et al.*, 1977; Barua *et al.*, 1997).
- Further there it also reduced the level of histamine and raised the plasma cortisol in antigen challenged guinea pigs (Tripathi and Shukla, 1979) as well as in bronchial asthma patients (Tripathi *et al.*, 1978). Das *et al.*, (2003) and Pramanik *et al.*, (2005) previously reported the anti-inflammatory activity of the methanol extract of *Albizia Lebback* bark. Many saponins, such as lebbekanin A-H (Varshney and Khan, 1961; Varshney and Sharma, 1969; Varshney *et al.*, 1973, 1976) and Albizziasaponin A-C (Pal *et al.*, 1995), which contain oleanolic acid, echinocystic acid or acacic acid as sapogenins were reported from various parts of this plant. Further, melanoxetin okenin-3-one, (+) pinitol, (-) leucopelargonidin (Gupta *et al.*, 1966).
- Alternative medicine for the treatment of various diseases is getting more popular. Many medicinal plants provide relief of symptoms comparable to that of conventional medicinal agents (Verpoorte, 1999).

4.5. *Albizia procera* (Roxb.) Benth.

Synonym: Mimosa procera Benth

The habitat ranges from monsoon forest, mixed deciduous forest, savannah woodlands, pyrogenic grasslands, roadsides and dry gullies, to stunted, seasonal swamp forest. It is commonly found in open secondary forest. It is a large deciduous tree. Leaves bi-pinnate with a large

gland near the base of the petiole, leaflets rigidly sub coriaceous, grey beneath, glabrous obliquely truncate at the base. Flowers whitish in copiously panicked heads. Pods thin, brown, glabrous. White siris is a large, fast growing tree with an open canopy that is almost evergreen but becomes leafless for a short time in the dry season. It grows up to 30 meters tall. The bole can be straight or cooked; it can be branchless for up to nine meters and up to sixty cm in diameter. An ornamental tree, it is often planted along avenues and in gardens to beautify them. Phenology is in May-June and September-March. In Katarniaghat Wildlife Sanctuary it is found both in miscellaneous forest and Sal forest with IVI of 2.8 and 0.5, respectively.

Ethno-botanical potential

- The tree is extensively harvested from the wild for its timber, many natural forests being managed on a forty year rotation.
- The tree is also grown as a plantation crop in Asia, Africa and the Americans.
- Fuel wood plantations are managed on a 20-year rotation.
- The cooked leaves are eaten as a vegetable. In times of scarcity the bark can be ground into a powder, mixed with flour and eaten.
- The tree is widely planted for its good soil binding capacity.
- It is occasionally cultivated as shade tree for tea and coffee plantations, where it also acts as a wind and fire-break.
- It is popular for the rehabilitation for seasonally dry, eroded and degraded soils. Its ability to grow on dry, sandy, stony and shallow soils makes it a useful species for reforestation for difficult sites.
- Good survival and rapid early growth have been reported in reforestation trials on both saline and alkaline soils, which are widely cultivated in agro-forestry system.

- The bark can provide tanning material. It is used in India for tanning and dying. However, its low tanning content (12-17%), considerable weight loss in dying and difficult harvesting has limited its importance.
- When injured, the stem exudes large amounts of a reddish-brown gum that is chemically similar to, and used as a substitute for, gum arabic (obtained from *Acacia senegal* and other species).
- The leaves are known to have insecticidal and pesticidal properties.
- The branches (twigs) are used by tea planters as stakes for lying out tea gardens. These are found to split well. The species is popular along field borders.
- Pods and fallen leaves should be considered not as undesirable litter but as potential energy sources. It seems probable that if the pods of the related species *A. lebbeck* can yield ten barrels of ethanol per hectare, then this species could as well.
- The timber has large amount of non-durable, yellowish-white sapwood.
- The heartwood large and heavy, light or dark brown with light and dark bands. Due to the broadly interlocked nature of the grain, it is more suitable for use in large section where a bold effect is desired, such as in large-sized panels and tabletops.
- It seasons and polishes well. The wood is used chiefly for construction, furniture, veneer, cabinet work, flooring, agricultural implements, moulding, carts, carriages, cane crushers, carvings, boats, oars, oil presses and rice pounders. It is resistant to several species for termites.
- The chemical analysis of the wood indicates that it is a suitable material for paper pulp. Bleached pulp in satisfactory yields (50.3%) can be prepared from *A. procera* wood by the sulphate process. It is suitable for writing and printing paper (mean fi-

bre length is 0.9 mm, mean fibre diameter is 0.021mm).

- The calorific value of dried sapwood is 4870 kcl/kg, and that of heartwood 4865 kcl/kg. An excellent charcoal (39.6%) can be prepared from the wood, and it is widely used as a fuel.

Ethno-medicinal potential

- White siris is commonly used in traditional medicines. Some research has been carried out into the medicinal activities of the plant and a number of active compounds have been recorded.
- All parts of the plant are anti-cancerous.
- The roots contain alpha-spinasterol and a saponin that possess spermicidal activity at a dilution of 0.008%.
- A decoction of the bark is given for the treatment of rheumatism and haemorrhage.
- It is also considered useful in treating problems of pregnancy and for stomach-ache. The leaves are poultice on to ulcers.

4.6. *Alstonia scholaris* (Linn.) R.Br.

Synonym: Echites scholaris Linn.

Alstonia scholaris found in India, Sri Lanka, Pakistan, Nepal, Thailand, Burma, South East Asia, Africa, Northern Australia, Solomon Islands, and Southern China. *Alstonia scholaris* is an evergreen tropical tree up to 80 ft in height, having greyish rough bark with lenticels, secreting white milky latex-rich in poisonous alkaloid, lateciferous which is bitter in taste. Leaves grow in clusters of seven, coriaceous, elliptic-oblong, 10 to 20 cm long, 3 to 4.5 cm wide, pointed at the base, rounded at the apex, glossy green on the upper surface, white or greyish on the underside. The tip of the leaf is rounded or shortly pointed, tapering towards the base. The blokes of larger trees are strongly fluted to 10 m. the outer blaze is cream to yellowish in colour with abundant, milky latex that flows rapidly when cut. The inflorescence is a much-branched

terminal panicle, up to 120 cm long; flowers 7-10 mm long white, cream or green, the tube hairy, lobes sparsely or densely pubescent, 1.5-4 mm long, the left margins overlapping, strongly perfumed. The fruits are thin pods that can grow up to 20 inches long. Fruit is made up two slender follicles which are pendulous and cylindrical follicles, 20 to 40 cm long, 4-5 mm in diameter. Seeds are 3 to 4 mm long, with brown ciliate hairs on the ends. Phenology- December-June. In Katarnia Ghat Wildlife Sanctuary it is found only in miscellaneous forest with IVI 0.4.

Ethno-botanical potential

The wood is too soft for making anything- so it is usually used in making packing boxes, blackboards, etc. *Alstonia scholaris* tree has been used to make paper.

Ethno-medicinal potential

- *Alstonia scholaris* has many medicinal properties like antimicrobial, anti-amoebic, anti-diarrheal, antihypertensive, anti-malarial, febrifuge, stimulant, hepatoprotective, immunomodulatory, anti-cancer, anti-asthmatic, antioxidant, analgesic, anti-inflammatory, anti-fertility, anti-diabetic, etc.
- *Alstonia scholaris* used in the treatment of fevers, chronic diarrhea, dysentery, ulcers, rheumatic pains, cancer, malarial fever etc.
- The ripe fruit of the plant are used in syphilis and epilepsy.
- The milky juice of *Alstonia Scholaris* has been applied to treat ulcers.
- The bark of the *Alstonia scholaris* is used in Ayurvedic medicine to treat fever, malaria, troubles in digestion, tumors, ulcers, asthma and so forth.
- The leaves and the latex are applied externally to treat tumors.
- The dried leaves of the *Alstonia scholaris* are used as an expectorant.
- The leaves can be used to treat skin diseases.

- The bark and roots are boiled with rice and eaten by girls daily for several weeks to treat excessive vaginal discharge.
- The roots and bark are used in traditional medicine as an anthelmintic, astringent tonic, alternative antidiarrhoeaticum, antiperiodicum, etc.
- The latex is used to clean wounds and can be used for chewing gum.

4.7. *Bombax ceiba* DC.

It is a large deciduous tree. Leaves are digitate, leaflets 5-7, flowers red or yellowish, capsules ovoid. Phenology is March-April. In Katarniaghat Wildlife Sanctuary is being found only in miscellaneous forest with IVI 11.00.

Ethno-botanical potential

- The silk cotton tree is often referred to as the silent doctor for the host of medicinal benefits that it offers almost each part of the tree, including the bark, flowers, fruits, seed and leaves, gums, thorns have therapeutic potential.
- A herbal composition made from the bark of the tree, for example is administered for the treatment of male sexual and gastro-intestinal disorders like dysentery and diarrhoea. The pharmacological benefits are basically due to the presence of Glycosides and tannins in the root and stem.
- It has haemostatic properties and is administered during menorrhagia.
- Silk cotton extracts are used in eye care, tentax forte, acne pimple cream. The plant is also being used for general debility, diabetes, impotence, spermatorrhoea, urinary stones and liver disorders.
- Some of the diseases for example diarrhoea, dysentery, asthma, rheumatism, leprosy, leucorrhoea, body pain, wounds are included in anti-inflammatory, analgesic, antimicrobial and oxytocic activities of plant as indirect evidence of

scientific validation (Jain and Verma, 2014).

4.8. *Diospyros cordifolia* Linn.

It is a large shrub or small tree. Leaves are ovate-oblong, ovate lanceolate, acute, base cordate or rounded and hirsute on both surface. Flowers pale white in axillary cymes. Fruits are globose yellow at maturity. Phenology-March-June and June-September, In Katarniaghat Wildlife Sanctuary is being found both in Sal forest and Teak Plantation forest with IVI of 3.2 and 3.0 respectively.

Ethnobotanical potentiality

- It is of great use for human being.
- It has commercial value being used in Bidi industry as a raw material.
- Leaves are being used in stupefying fishes.
- It is being used in several ailments either as a cure or for the well-being, viz., used for lever disorders, whooping cough, leprosy, ulcers, gonorrhoea, fever as emetic and anti-helminthic. Alcoholic extract are anti-inflammatory, antipyretic and analgesic. It is depressant, spasmolytic producing bradycardia and hypotension. Aqueous extract is being used in critical jaundiced condition.
- The fruits are consumed because of its juicy and sweet nature by local inhabitants (Mall, 2016).

4.9. *Ficus racemosa* Linn

Synonym: *Ficus glomerata* Roxb.

A large deciduous tree, buttressed at the base. Bark smooth reddish brown; blaze pink, fibrous with white latex turning yellow on exposing. Leaves are 5-15 x 2.5-6.5 cm, alternate, ovate or elliptic-lanceolate, entire, sub-acute, base rounded or acute, glabrous above, minutely dotted beneath. Receptacles 2-3.2 x 2-3.5 cm, clustered on leafless branches, smooth or pubescent, red or pink at maturity. Plant is propagated by using cut-

tings of stem and root shakers or by seeds also. The flowers are pollinated by very small wasps. Phenology: April – July. In Katarniaghat Wildlife Sanctuary is being found both in miscellaneous forest and Teak Plantation forest with IVI of 9.7 and 1.9, respectively.

Ethnobotanical potentiality

- Traditionally it is used in Indian medicinal practice as astringent, carminative, stomachic, vermicide etc (Mall and Tripathi, 2017).
- The extract of fruit is used in leprosy, diarrhoea, menorrhagia. It is useful in the treatment of leucorrhoea, blood disorder, burning sensation, fatigue, urinary discharge, intestinal worms and as carminative.
- Leaves are astringent to bowels and good in case of bronchitis; leaves are used in dysentery young tender leaves are used for fair complexion. The decoction of leaves is used to wash the wounds and ulcers.
- Bark is useful in asthma and piles. The latex or milky juice is administered in chronic infected wounds, haemorrhoids, boils, traumatic swelling, toothache, vaginal disorder, wounds it promote healing very soon.
- The root sap is used for treating diabetes.
- Phytochemical properties: The leaf of this plant contains sterols, triterpenoides (lanosterol) and alkaloids, tannins and flavonoids. Stem bark gives gluanol acetate, β -sterol, lupenol, stigmasterol. Fruit contains gluanol acetate, glucose, tiglic acid, esters of taraxasterol, lupeol acetate and other phytosterols.

4.10. *Madhuca latifolia* Roxb.

Madhuca is a large deciduous tree reaching a height up to 20m. Leaves are large and broadly elliptic 12-20cm long. The bark is 1.2 cm thick. Flowers white to cream colour with tubular, fleshy and juicy corolla. Fruit berry, ovoid, green

at maturity and turn pinkish yellow when ripe.

Ethnobotanical potentiality

- The madhuca contains protein, carbohydrate, fat, minerals, calcium, phosphorus, iron, carotenes, sugar. Vitamins and many other nutrimental chemical constituents.
- The bark of Madhuca is used to cure leprosy and to heal wound.
- The flower decoction is used for headache due to cough and cold.
- Paste of fresh bark is useful on joint and muscles pain.
- Whole plant decoction is taken orally which is useful in joint and muscles pain (Mall and Tripathi, 2017a).

4.11. *Syzygium cumini* Skeels.

It is a large evergreen tree with whitish brown bark. Every year the bark is shed off. Its leaves are simple pointed at the tip, somewhat leathery, oval to rectangular and somewhat shiny. Flowers are mostly white and appear in cluster from axil to leaves. The fruit is berry.

Ethnobotanical potentiality:

- The fruit contains 88% moisture, 0.7% protein, 0.1% fat, 19.7% carbohydrate and 0.4% minerals. Fresh fruit had the antioxidants 708 mg/100g AEAC units. The ripe fruit contains anthocyanin pigment (Rao *et al.*, 2006). *Syzygium cumini* is a well-known anti-diabetic herb.
- It is a good immune modulator. It is also used in blood pressure, dysentery, diarrhoea and gingivitis.

4.12. *Schleichera oleosa* (Lour.) Oken.

Synonyms: *Pistacia oleosa* Lour. *Schleichera trijuga* Willd., *Cussambium oleosum* Kuntze., *Melicocca trijuga* Juss.

It is a monotypic genus belonging to the same family to which the popular fruit 'Litchi' belongs. The generic name of kusum, *Schleichera* is derived after the Swiss botanist J. C. Schleicher who first described the tree. The species name ole-

osa is derived from the Latin word 'oleosa' meaning oil, as the seed kernels are rich in oil. Synonymously the tree is also referred as *Schleichera trijuga* Willd., the word trijuga stands for 'three pairs', based on the presence of three pair of leaflets in a leaf. Kusum is a large forest tree with dense green foliage. Leaves pinnate with three pairs of leaflets. Inflorescence raceme. Flowers are white and fruits small. The fruits are berry shaped, globose or ovoid with a hard skin. The seeds are brown, irregular elliptic, slightly compressed oily and enclosed in a succulent aril. The oil content of the seed is around 59-72% with yellowish green color. Phenology is in October-November.

It is locally known as kusum. The other common names are kusum, kusumb, kosumb, koshamara, Celon oak kosamara, lac tree, honey tree, gum lac tree, macassar oil tree, sukoshka, skrataka, jatudruma, koshamra, jantu vriksha and kshudra maukkuli, etc. It occurs in the Indian sub-continent and south East Asia. There are many trees that are grown for multiple products. They are known as multipurpose trees (MPTs), a term widely used in agro-forestry. Kusum is also one among the multipurpose trees which has been proved to be useful in numerous ways from times immemorial.

In Katarniaghat Wildlife Sanctuary (KWS) it occurs in all the three types of forests with different IVI values. In miscellaneous forest, Sal forest and Teak plantation the IVI value of the kusum plant is 1.5, 4.3 and 4.4, respectively.

The available literature reveals that this multipurpose ethno-botanical, nutrimental, ethno-medicinal, ethno-veterinary and plant of environmental use in agro-forestry which provide shade, habitat for organisms, soil improvement, etc., many useful products are also obtained such as fruits, timber, fire wood and variety of metabolic chemicals which may be used in the form of home remedies and for traditional medicine. Considering the multipurpose importance of the tree, which is yet not popularise due

to one reason or the other despite providing an array of benefits (Mall and Tripathi, 2017b).

Ethno-botanical potential

- The leaves, twigs and the seed- cake are used as fodder to feed cattle.
- The wood is suitable as firewood and makes excellent charcoal.
- Pressed oil cakes from kusum tree are rich source of crude protein, carbohydrate, fibre and other minerals and serves as nutritive cattle feed.
- The oil extracted from the seed, called as kusum oil is used for culinary and lighting purposes.
- The kusum oil is being used to cure itching, acne, burn and other skin problems.
- The oil is used in rheumatism by external massage.
- Kusum oil is used in hair dressing as well as for promoting hair growth.
- The pinkish-brown heart wood is very hard, durable and excellent to make pestles, cartwheels, axles, plows, tool handles, and rollers of sugar mills and oil presses.
- Kusum plant is known for lac cultivation. It is one of the major host plant commercially exploited for cultivation of the Indian lac insect (*Kerria lacca*). It supports the kusmi strain of lac insect, which produces good quality, natural, biodegradable and commercially important, light colored lac resin of demand by lac industry, thus fetching high remunerative prices to lac growers. The lac resins serves as a livelihood support to millions of poor farmers in states like Jharkhand, Chattisgarh, Orissa, Andhra Pradesh and West Bengal. Immature lac insects prefer semi-tender twig of kusum tree for sap sucking and start secreting resins surrounding their body. The resinous coatings of closely settled sessile insects eventually coalesce together to form an encrustation in five to six months. On kusum tree, two lac crops are pro-

duced in winter and summer season. About 34-38% of the total lac production of India is shared by the Kusum tree as lac host. There are also other lac hosts but the quality of kusum lac is far superior. The dense foliage of the mature kusum tree provides an additional advantage of supporting brood lac (inoculums stick lac with emerging larvae from the female resin cells) viability even during the very hot summer season, otherwise summer mortality of lac insects is a common problem with other host plants like *Butea monosperma* (palas) and *Ziziphus mauritiana* (ber).

- The seeds of kusum are a very rich source of oil (60-72%) for industrial implications. The seed oil called kusum oil is an important component of the Makassar oil used for hair dressing and cooling bath oil.
- Kusum oil is used in textile industry for batik applications and also for making soap.
- The bark of kusum tree produces tannins and dyes that are occasionally used in small-scale industries like tanning in leather industry.
- Young leaves and shoots-raw cooked in soups or steamed and served with rice.
- The ripe fruit is eaten raw which has a pleasant acid flavor.
- The unripe fruits are pickled.
- Oil obtained from the seed called macassar oil, is sometimes used for culinary purposes. It contains cyanogenic compounds, which may cause giddiness and should be removed if the oil is used for human consumption.
- The kusum tree is also grown as an avenue tree or wayside tree.
- The tree is utilized for multifarious purposes and is a boon for a subsistence farmer.
- The extended foliage and canopy of the kusum tree provides good shade and is therefore, suitable for mixed

farming with other heat susceptible economic plants.

Importance of S. oleosa as a biodiesel fuel

The depletion of the conventional petroleum resources has become a problem of major concern in recent years. Extensive research is going on to find an alternative fuel. Since vegetable oils have properties similar with that of diesel, they are replacing diesel in the field of commercial transportation and agricultural machinery. But the direct use of vegetable oil is having adverse effects on the combustion engine. Therefore, these vegetable oils are converted to biodiesel. Blending, emulsification, thermal cracking, and trans-esterification are the few techniques used for the conversion of crude vegetable oil into biodiesel. At present, biodiesel is produced by sunflower oil, palm oil and soybean oil by trans-esterification process. These oil due to their non-toxic, biodegradable and renewable nature, have gained a lot of alteration by the researchers. Cetane number for biodiesel is higher than that of petroleum. Moreover, biodiesel does not contain aromatic components. The emission of carbon monoxide, hydrocarbon and particulate matter is also less as compared to that of diesel fuel. High cost of the above mentioned oil is the basic disadvantage associated with them. Hence, the non-edible type of oils yielded from trees such as mahua, sal, in-seed, castor, karanja, neem, rubber, jatropha, kusum, cashew, restaurants waste oils and greases along with animal fats are best suited for the production of biodiesels, for instance, *S. oleosa* seed oil, one of the many non-edible seed oil is found to have many cyanogenetic materials and free fatty acids (FFA) such as myristic acid, palmitic acid, palmitoleic acid, cis oleic acid, trans linoleic acid, cis linoleic acid, alpha linolenic acid, eicosadienoic, heneicosanoic, behenic acid, erucic acid, lingoceric acid, docosahexaenoic acid (Mikolajczek and Smith, 1971, Canacki and Gerpen, 2001

and Gandhi *et al.*, 2011). Therefore, it is used in production of biodiesel. In a report by Gandhi *et al.*, 2011 methyl ester was produced using *S. oleosa* seeds.

Phytoremediation properties

- *Callophyllum inophyllum* L. and *Bixa orellana* L. (Chaturvedi *et al.*, 2012).
- Mining, smelting of metalliferous, dumping of waste, chemicals used in agriculture etc. are the different source of soil pollution, but the waste rocks generated by mining is the main source of the metal pollution of soil. The direct consequences of the deposition of waste rocks on the surface are the loss of cultivatable lands, forest and grazing land (Clemente *et al.*, 2007, Rio *et al.*, 2006 and Freitas *et al.*, 2004). Activity such as grinding, crushing, washing and smelting, used to extract and concentrate metals, generate waste rocks and tailings. Most of the tailings exhibit acidic pH due to which the microbial activity decreases which in turn leads to the death of plants. Tailings do not contain organic matter and are characterized by the high concentration of arsenic, cadmium, copper, manganese, lead, zinc and other heavy metals (Mukhopadhyay and Maiti, 2010). However some plants can exist in the region of high concentration of metals (Das and Maiti, 2008). Such plants can be used to restore the contaminated sites by the process of phytoremediation. Phytoremediation is an environmental friendly and cost efficient technique used to treat the contaminated soil, air or water through the use of plant without employing any soil excavation or mechanical clean up method. Although many physico-chemical techniques are also available to extract metals such as acid leaching and electro-osmosis, but these techniques are quite costly and can decontaminate only small portions of land (Ramadhas and Murleedharan, 2005). Moreover, these techniques also dete-

riorate biological activity of the soil and adversely affect its physical structure. Therefore, the phyto-remediation is the preferred technique to decontaminate the soil. This approach to remove the metal is called green mining because further extraction of metals can be done from the plant tissue (Clemente *et al.*, 2007).

Use as livestock feed

- A few non-conventional agro-industrial by-products including *S. oleosa* cake were checked for their effectiveness as a livestock feed (Punj, 1988). The presence of tannins adversely affects the utilization of various nutrients (McLeod, 1974). In addition, tannins are believed to create toxic effects by breaking down the alimentary canal tissues and the hydrolysable tannins make pathological changes in liver, kidney, heart, etc. When their concentration in blood increases further than the competence of the liver to deify them. The levels of tannins were determined using various chemical and biological methods. It was observed that in *S. Oleosa*, tannin levels in terms of total phenols (TP) and condensed phenols (CP) were low, and protein-precipitation capacity (PPC) could not be detected because of its very low level. Hence, it can be considered safe for incorporation in livestock feed since the harmful factors are absent (Makkar, 1990).

Ethno-medicinal potential

- Different plant parts (stem bark, seed, fruit and seed oil) of kusum are used in traditional medicines.
- The seed oil is used by the local vaids for curing skin diseases like scabies, itching, and acne.
- The bark decoction is also used against skin inflammation and ulcers.
- The bark decoction is also infused for curing malaria.
- The fine paste of the bark of Kusum is often used to control tissue swelling.

- The bark is known to contain medically important compounds like lupeol used in preparing analgesic and antitumor agents like betulin and betulinic acids.
- It balances kaph, useful in productive cough and asthma.
- It cleanses intestine.
- It is used in bleeding disorders like nasal bleeding and heavy periods.
- The unripe fruits are astringent, useful in diarrhea, neuralgia, paralysis, constipation and bloating.
- The fruit pulp improves hair strength and promotes hair growth.
- The ripe fruit improves digestion strength, improves taste and relieves anorexia.
- The leaf, seed, oil, and bark are used for treating rheumatoid arthritis, headache, myalgia, skin disease, malarial fever and prophylactic against cholera.
- The bark is astringent and is used in fever as antipyretic, useful in pruritus.
- The kusum oil is bitter, sour, sweet which improves strength and immunity and can be taken regularly. It improves taste and relieves anorexia.
- The oil is digestive, induces mobility, causes diarrhea, purgative and relieves constipation.
- The fine paste of the bark which is astringent is mixed with oil and applied to cure itch and acne and other skin eruptions.
- The oil is useful in worm infection, skin diseases, in toxic conditions, poisoning, ulcer and wounds.
- The seed oil is also used for the cure of itch and acne.
- The seed oil is stimulating and has cleansing applications.
- The ripe fruit is often served with salt which improves digestion, useful in anorexia and nourishing.

Ethno-veterinary potential

- The seed is grinded so as to make fine powder. It is mixed with wa-

ter and given to cattle for removing worms from the stomach.

- The fine powder of the seeds is applied to wounds and ulcers of cattles to remove maggots.

Phyto-chemical constituents

Phytochemical studies have shown that its bark contains lupeol, lupeol acetate, betulin, betulinic acid, beta-sitosterol, and scopoletin (Dan and Dan, 1986). A very recent report have also shown the existence of taraxerone and tricadenic acid A in the outer bark of the above plant (Ghosh *et al.*, 2011). The bark also contains about 10% tannin and antitumor agents such as betulin and betulinic acid have also been isolated from it.

Anticancer activity

Cancer is a term used for a disease in which abnormal cells trend to proliferate in an uncontrolled way and, in some cases metastasize. Extensive research has been done in order to find therapeutic drug for the treatment of cancer. Plant based products have been frequently examined as potential anticancer agents. The screening of various medicinal plants results in the isolation of bioactive compounds which have been reported as effective chemopreventive as well as chemo therapeutic agents (Kawamori *et al.*, 1999, Choi *et al.*, 2001, Kirana *et al.*, 2003 and Sandhya *et al.*, 2006). The phytochemical screening of *S. oleosa* revealed the presence of lupeol and butilinic acid type triterpene which have antineoplastic activity (Bhatia *et al.*, 2013). This study provides a step toward the exploration of *S. oleosa* as a chemo preventive agent against cancer. A bulk of research revealed that the phyto-chemicals exhibit their anticancer properties either by suppressing the proliferation of tumor cells via suppression of various cell signalling pathways or by induction of apoptotic death in tumor cells by generation of free radical, such as reactive oxygen/nitrogen species (Bharti *et al.*, 2003 and Pettit *et al.*, 2000). A report involving the separa-

tion of an extract prepared from the bark and stem of Sri lankan tree *S. oleosa* results in the isolation of seven sterols, Scheicherastins (1-7) and two related sterols 8 and 9 designed as Schleicheols 1 and 2. The isolated Scheicherastins exhibited cancer cell growth inhibitory properties (Pettit *et al.*, 2000).

Antioxidant activity

Oxygen is used for generating metabolic energy in our body but it also produces reactive oxygen as by products during its various reactions in the body. Reactive oxygen species are usually atoms or a group of atoms having odd (unpaired) electrons, in aerobic cells these are produced during mitochondrial electron transport and several oxidation reactions (Forman and Torres, 2002). These reactive species can react with DNA and several other bio-molecules causing what is called 'oxidative damage to DNA' this damage causes changes in DNA such as strand breaks; changes at cross links between DNA and protein; changes at base pair sites among other changes (Dizdaroğlu *et al.*, 2002). Several medicinal plants, fruits, vegetable can decrease the risk of oxidative damage as they comprise of vitamins, carotenes, phenolic compounds, flavanoids, alkanoids, tannins, etc. which act as chemo-preventive agents (Dhir *et al.*, 1993, Cozzi *et al.*, 1997 and Thind *et al.*, 2012). These phytochemicals can prevent damage by their radical scavenging ability. Thind *et al.* evaluated the hydroxyl radical scavenging potential of *S. oleosa*. Extracts of roots of *S. oleosa* with different solvents were tested for their anti-proliferative activity.

Antioxidants are molecules which can safely interact with free radicals and terminate the chain reaction before vital molecules get damaged. The free radical damage can be prevented by several enzymes and the principle antioxidants such as vitamin E, beta-carotene, and vitamin C, present in the defence system of our body. Several studies have shown that plant phenolics also have antioxidant

properties (Velioglu *et al.*, 1998, Maisuthisakul *et al.*, 2007 and Li *et al.*, 2008). Natural polyphenols can have simple structure for example phenolic acid, phenylpropanoids, flavonoids can have simple structures like polymers, *e.g.*, lignin, melanins, tannins (Bravo, 1998). Free radical scavenging property, metal chelating property, effects on cell signalling pathways and on gene expression contributes to the potential of phenolics as antioxidant therapeutic agents (Soobrattee *et al.*, 2003). *S. oleosa* has been found as potent antioxidant due to the presence of phenolic compounds (Thind *et al.*, 2011).

Antimicrobial activities

In recent study two (Ghosh *et al.*, 2011) triterpenoids, namely taraxerone and tricadecic acid A were isolated from the outer bark and preliminary study on their antimicrobial activities were done against five different fungal pathogens namely *Colletotrichum camelliae*, *Fusarium equiseti*, *Altermaria alterata*, *Curvularia eragrostidis*, *Colletotrichum gloeosporioides* by in vitro antifungal assay (Suleman *et al.*, 2002 and Saha *et al.*, 2005) and against four bacterial pathogens namely *Escherichia coli*, *Bacillus subtilis*, *S. aureus* and *Enterobacter* by antibacterial assay. It was found that both taraxerone and tricadecic acid A had prominent activities against the fungal and bacterial pathogens.

The enumerations collectively show the various pharmacological activities of *S. oleosa*. It has potential of anticancer, antioxidant and antimicrobial activities. It contains various poly phenolic compounds. The poly phenols scavenge free radicals and do not allow them to damage the cell. Due to its free radicals scavenging activity, *S. oleosa* is a potent antioxidant. Free radical scavenging activity can also be correlated to cytotoxicity. It exhibits toxicity against various cell lines and was found to be as effective anticancer agent. It, moreover, has a great scope of being an effective antimicrobial agent since it showed good ac-

tivity against various microbes. It was also found that this plant has various environment aspects to it as well. The biodiesel produced from it. Is found to have many properties similar to that of diesel *e.g.* viscosity and volatility. Also its cetane number is higher than that of petroleum; therefore it can replace diesel for the combustion engine. On the basis of physic-chemical, growth and biochemical parameters *C. inophyllum* and *B. orellana* were found to be more capable for phyto-remediation of the contained soil compared to *S. oleosa*. Furthermore, it was observed that it contained low tannin levels, thus it can be considered safe to be used as a livestock feed.

The major use of kusum tree is for cultivation of lac. However, other uses of kusum tree are currently underexploited but hold promise to benefit human life in many spheres. The plantation of kusum in suitable areas needs to be promoted in view of its advantages as MPT. There is a tendency to prefer quick growing trees in the forestation programmes, which may not always be advantageous or even eco-friendly in long run.

The role of kusum plantation can mainly be envisaged in terms of economic benefits to the resource-constrained farmers dwelling around forest areas.

4.13. *Ziziphus mauritiana* Lam.

Ziziphus mauritiana is an extremely drought hardy and native fruit of India, found wild and cultivated.

Ethnobotanical potentiality

It is useful as food, fodder, nutrient, medicinal, construction material and fuel.

Z. mauritiana is having tremendous medicinal properties, attributed by diverse group of secondary metabolites such as alkaloids, flavonoids terpenoids, saponin, pectin, triterpenic acids and lipids. Jujubosides (saponin) isolated from *Ziziphus* reported to have haemolytic, sedative, anxiolytic, and sweetness inhibiting properties. Whereas, cyclope-

tide alkaloids, found to have sedative, antimicrobial, hypoglycaemic, anti-plasmodial, anti-infectious, anti-diabetic, diuretic, analgesic, anticonvulsant and anti-inflammatory activities (Goyal *et al.*, 2012).

In spite of the fact that *Ziziphus mauritiana* having medicinal properties it is neither considered as important medicinal plant nor utilized for medicinal use in main stream therapeutic.

From the present study, it is envisaged that the trees of *Katarniaghat* Wildlife Sanctuary has great socio-economic importance as they are being widely used for different purposes by the natives. Nature has provided a complete store house of remedies to cure ailments of mankind. Besides, traditional and commercial importance, they have tremendous ecological significance. Trees which are of leguminous nature and soil binding abilities, they all are suitable species for wasteland development. However, other uses of many trees are currently underexploited but hold promise to benefit human life in many spheres. The plantation of plants in suitable areas needs to be promoted in view of their advantages as malty purpose trees (MPT). There is a tendency to prefer quick growing trees in the forestation programmes, which may not always be advantageous or even eco-friendly in long run. The trees must be conserved and more plantations should be done either by utilisation of Biotechnology or through traditional methods. People conserve what they love. They love what they understand and they understand what they are taught.

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Free Radical Scavenging Potential and Anticancer Activity of *Primula denticulata* Sm. from North-Western Himalayas

Bilal Ahmad Wani^{1,*}, Mohammed Latif Khan¹ and Bashir Ahmad Ganai²

¹Department of Botany, Dr. Hari Singh Gour Central University, Sagar, India- 470003

²Centre of Research for Development, University of Kashmir, Srinagar, 190006, India;

*Correspondence: bilalenvsci@gmail.com; Tel: +91-9407579802

Abstract: The present research work was aimed to evaluate the free radical scavenging potential and anticancer property of *Primula denticulata* ethanol extract (PDEE) against different human cancer cell lines. The antioxidant potential was determined by total phenolic and flavonoid content, DPPH free radical scavenging assay, hydroxyl radical scavenging assay and DNA damage assay. The anticancer activity was determined by SRB assay. Apoptotic induction in MiaPaca-2 cells was analysed by propidium iodide staining cell-cycle and DNA content analysis, and mitochondrial membrane potential loss was measured using rhodamine-123 as fluorescent dye through flow cytometry. The total phenolic and flavonoid content in PDEE was found to be 12.24±2.11 (mg GAE/g dry extract) and 7.06 ±1.31 (mg catechin/g dry extract) respectively. The extract at 600 µg/ml concentration induces 69.35% DPPH free radical inhibition. In hydroxyl radical scavenging, the extract showed 54.51% inhibition at 120 µg/ml concentration. PDEE prevents DNA damage against oxidative stress in concentration dependent manner. Anticancer activity was evaluated against six human cancer cell lines (MiaPaca-2, A-549, PC-3, THP-1, HCT-116 and HOP-620). The extract showed significant anticancer activity in concentration dependent pattern. The highest activity was shown against MiaPaca-2 cell line. PDEE induces significant apoptotic induction in cells. Exposure of MiaPaca-2 cells to PDEE (0-100 µg/ml) caused dose dependent cell cycle arrest at G₀/G₁ phase and induced apoptosis by increasing accumulation of cells at G₀/G₁ phase. PDEE increased the apoptotic cell population from 11.4% in case of control to 49.6% at 100 µg/ml. Further, PDEE induces loss of mitochondrial membrane potential ($\Delta\Psi_m$) to 99.5% at 100 µg/ml from 24.4% in control cells. These primary results depict the free radical scavenging potential and anticancer activity of *P. denticulata* extracts. These findings may serve as foundation to develop an anticancer drug from medically important *P. denticulata*.

Keywords: Apoptosis; DNA damage; DPPH; MiaPaCa-2 cells; *Primula denticulata*

1. Introduction

Cancer after cardiovascular diseases is the second leading mortality cause and is rapidly becoming a global pandemic. The worldwide incidence and mortality of cancer in 2008 were 12.66 and 7.56 million cases respectively. According to (World Health Organization, 2010) report, the global cancer burden is expected to nearly double to 21.4 million

cases and 13.5 million deaths by 2030. Pancreatic cancer is the fourth most common cause of cancer-related deaths across the world with incidence equalling mortality and continues to pose an enormous challenge to clinicians and cancer scientists (Hariharan *et al.*, 2008). Among all pancreatic cancers, pancreatic ductal adenocarcinoma (PDAC) is the most common epithelial, exocrine pancreatic malignancy, representing more than 80%

of the malignant neoplasms of the pancreas (Alexakis *et al.*, 2004).

Consumption of fruits and vegetables is known to impart reduction in the incidence of ischemic heart disease and some types of cancer, particularly stomach, oesophagus, lung, oral cavity and pharynx, endometrial, pancreas and colon cancers (Mathew *et al.*, 2004). Similarly natural antioxidant supplements (ascorbic acid, tocopherols, anthocyanin, β -carotene and other polyphenols have been associated with lower incidences of cancers and cancer related diseases (Fleischauer *et al.*, 2003). During some pathophysiological conditions, excess amount of reactive oxygen species (ROS) is being generated by certain external agents such as UV-radiations, drugs, pollution, other xenobiotics and as well as by endogenous chemicals, especially stress hormones (adrenalin and noradrenalin). The superoxide dismutase (SOD) and other defence mechanisms in living organisms are unable to scavenge excess of ROS completely, which causes damage to cellular molecules such as DNA, RNA, enzymes, lipids etc. that results in fluidity of biomembranes (Dean and David, 1993) and development of degenerative diseases including cancers, cardiovascular, neurodegenerative, Alzheimer's and inflammatory diseases (Shahidi *et al.*, 1992; Gerber *et al.*, 2002; Di Matteo and Esposito, 2003; Sreejayan and Rao, 1996). Hence there is growing interest in natural polyphenolic compounds, present in medicinal and dietary plants that might help attenuate oxidative damage (Silva *et al.*, 2005).

The increased incidence of different types of cancers during the last few decades and the modern techniques for separation, structure elucidation, screening and combinatorial synthesis have led to the development of new anticancer drugs, drug combinations and chemotherapy strategies by exploration of enormous pool of biological, synthetic and natural products (Mukherjee *et al.*, 2001). So far several potential anticancer lead molecules such as taxol, vincristine, vinblas-

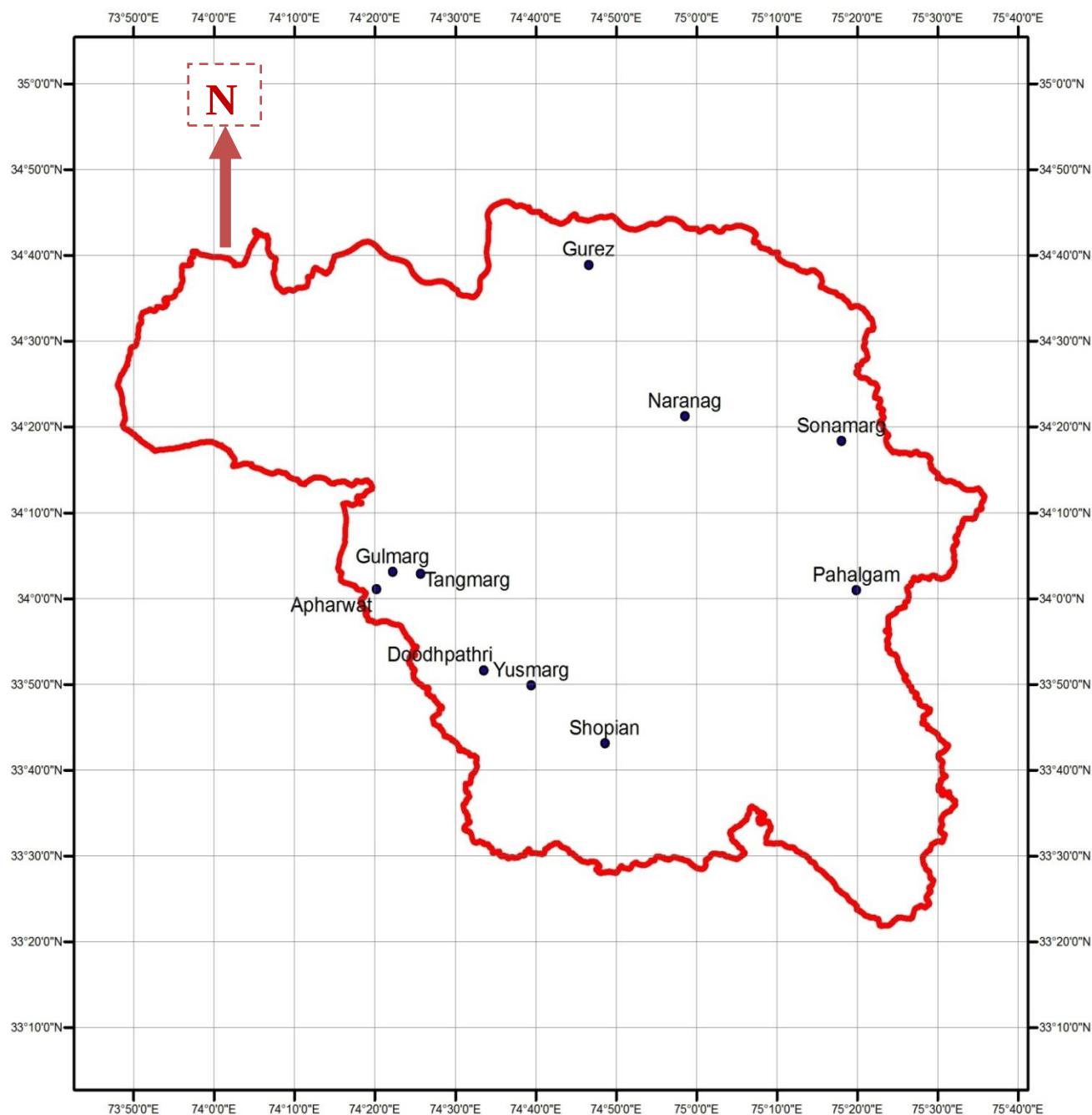
tine, podophyllotoxin, camptothecin, combretastatins, flavopiridol, bruceatin etc, with diverse chemical structures have been isolated from plants. Several biologically active analogues such as taxotere, isotaxel (taxol analogues) topotecan, irinotecan, rubitecan, lurtotecan, 9-Amino CPT (camptothecin analogues), etoposide, teniposide (podophyllotoxin analogues), vinorelbine, hydravin (Vinca alkaloid derivatives) have been synthesised from these front line anticancer lead molecules (Vandana *et al.*, 2005). As a result, emphasis has now been shifted towards the screening of apoptotic inducers from natural sources particularly from plants in the form of extracts or as isolated compounds that specifically increase apoptotic cell death in cancerous cells.

Primula denticulata Sm. (Primulaceae) is an important member of genus primula, which represent more than 400 species (Richards, 1993). *P. denticulata* is commonly known as drumstick primula or tooth-leaved primula. *P. denticulata* is 20-30 cm tall perennial rarely annual, deciduous, clump-forming plant with compact heads of many flowers. The plant is widely distributed from eastern Afghanistan and northern Pakistan, across the Himalaya to Yunnan, Sichuan and Guizhou in China. In Kashmir Himalaya, the species is widely distributed (Map-1). The species thrives best in moist, shady slopes, mostly near melting glaciers and moist meadows, ranging in altitude from 2100 – 4050 meters.

2. Materials and methods

2.1. Chemicals

Sulphorhodamine-B (SRB), RPMI-1640 medium, fetal bovine serum (FBS), streptomycin, sodium bicarbonate, 5-Fluorouracil, paclitaxel, gentamycin sulphate, trypsin, 1,1-diphenyl-2-picrylhydrazyl (DPPH), folin-Ciocalteu reagent, catechin, gallic acid were procured from Sigma-Aldrich. Trichloroacetic acid (TCA), butylated hydroxytoluene (BHT), thiobarbituric acid (TBA), hydro-



Map 1: Distribution of *Primula denticulata* Sm. in Kashmir Himalaya, J&K- India.

gen peroxide (H_2O_2), ferric chloride, dimethyl sulfoxide (DMSO), potassium ferri-cyanide were purchased from Merck. The other reagents used were all of analytical grade.

2.2. Collection and identification of plant

Primula denticulata Sm. at flowering stage was collected from Gulmarg region of Kashmir Himalaya (latitude $34^{\circ}3'27''$ N; longitude $74^{\circ}23'9''$ E) at alti-

tude of 2650 m. The healthy plant species were randomly collected by hand-picking and later identified by Dr. Anzar A. Khuroo at department of Botany, University of Kashmir. A specimen under voucher number KASH-1743 was preserved for future reference.

2.3. Extract preparation

Fresh and healthy leaves of *P. denticulata* (1Kg) were cleaned with dou-

ble distilled water, dried under shade ($25 \pm 2^\circ\text{C}$) for 5-6 days. The dried plant material was ground to powder form. The plant powder was extracted in soxhelt apparatus using ethanol as solvent at desirable temperature. The filtered extract was concentrated using Buchi rotavapour and stored in glass vials at 4°C until used.

2.4. Estimation of total phenolics

The total phenolic content in leaf extract of *P. denticulata* was determined by Folin–Ciocalteu method as adopted by Slinkard and Singleton (1977) with slight modifications. To 0.2 ml of plant extract (1mg/ml) was added to 2.5 ml of 10% diluted Folin–Ciocalteu reagent and 2 ml of 2.5% aqueous Na_2CO_3 . The reaction mixture was incubated at room temperature with intermittent shaking. The blue colour solution was read at 765 nm on UV–visible spectrophotometer. The absorbance of solution was compared against standard Gallic acid (50 mg %) calibration curve.

2.5. Estimation of total flavonoids

The aluminium chloride colorimetric method as described by McDonald et al., (2001) was used to determine the total flavonoid content of leaf extract. The principle of this method is based on flavonoid–aluminium complex formation, which shows absorbance maximum at 430 nm. Briefly 0.5 ml (1mg/ml) of extract was mixed with 1.5 ml of ethanol, 0.1 ml of 10% AlCl_3 , 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water. After 5 min of incubation, the absorbance was read at 430 nm. Flavonoid concentration was expressed as milligrams of catechin equivalents per gram dry weight.

2.6. DPPH assay

DPPH assay is one of the most extensively used method for determining the antioxidant potential of any biological sample. DPPH is a purple stable free radical which is reduced to yellow colour complex 1,1-diphenyl-2-picrylhydrazine (DPPH-H) by compounds which are ca-

pable of donating hydrogen or electron. 100 μl of different concentrations (100–600 $\mu\text{g}/\text{ml}$) of plant extract or standard antioxidant was added to 1 ml DPPH solution (0.5 mM). The solution was slightly shaken and kept stand for 30 min at room temperature under dark conditions. The yellow colour solution was read at 517 nm against ethanol (Brand-Williams et al., 1995). The free radical inhibition was calculated as:

$$\text{Percentage inhibition} = [(A_c - A_s)/A_c] \times 100$$

Where, A_c and A_s are the absorbance of control and sample respectively

Butylated hydroxytoluene and α -tocopherol were used as positive control.

2.7. Hydroxyl radical ($\text{HO}^\bullet$) scavenging assay

Deoxyribose assay was used to evaluate the hydroxyl radical scavenging potential of *P. denticulata* leaf extract (Halliwell et al., 1987). The $\text{HO}^\bullet$ generated in Fenton reaction attack deoxyribose to form products that upon heating with thiobarbituric acid at low pH yield a pink chromogen (TBARS). A reaction mixture containing deoxyribose (25 mM), FeCl_3 (10 mM), ascorbic acid (100 mM), H_2O_2 (2.8 mM) in 10 mM KH_2PO_4 (pH 7.4) with or without plant extract at various concentrations (20–120 $\mu\text{g}/\text{ml}$) and incubated at 37°C for 1h. Then 1 ml of TBA (1% w/v) and 1 ml of TCA (3% w/v) were added and heated at 100°C for 20 min. Absorbance of TBARS was read at 532 nm. Deoxyribose oxidation inhibition was calculated as:

$$\text{Percentage inhibition} = [(A - B)/A] \times 100$$

Where, A is malonaldehyde produced when treated with extract and B is malonaldehyde produced without extract. Butylated hydroxytoluene and α -tocopherol were taken as the positive control.

2.8. DNA damage assay

The Prevention of oxidative DNA damage by PDEE was determined by method as previously described by Ghanta et al., (2007). Calf thymus DNA

(0.37 µg) with and without plant extract (10, 30, 50, 80 and 100 µg) was incubated with 20 mM ferric nitrate 30 mM H₂O₂ in 20.0 mM phosphate buffer (pH 7.4) in a final reaction mixture volume of 20 µl for 1h at 37 °C. Oxidative DNA damage was induced by hydroxyl radicals generated in Fenton reaction (Ani *et al.*, 2006). Bromophenol blue (0.25%) and glycerol (30%) were added to terminate reaction mixture, followed by gel electrophoresis in 0.7% agarose. The gel was then visualized and photographed on gel doc.

2.9. Cells culture

Pancreatic cell line (MiaPaca-2), Lung cell line (A-549), Prostate cell line (PC-3), Leukaemia cell line (THP-1), Colon cell line (HCT-116) and Lung cell line (HOP-62) were purchased from National Cancer Institute, U.S.A and European collection of cell culture, UK. Cells were cultured in RPMI-1640 and MEM medium supplemented with nutrients and antibiotics. Cells were grown at 37 °C and 5% CO₂ level with relative humidity of 98%.

2.10. Anticancer activity

The anticancer activity of PDEE against different human cancer cell lines was evaluated by SRB assay as described by Monks *et al.*, 1991. Briefly 100 µl of cell suspension (1x10⁵ cells/well) were cultured in 96-well plates and incubated overnight at 37 °C and 5% CO₂ level. 20 µl test material at various final concentrations (10-100 µg/ml) was added. Paclitaxel (1 µM) and 5-fluorouracil (20 µM) were used as standard anticancer drugs. Cell growth was stopped after 48 hrs of incubation by adding 50 µl of 50% TCA in each well and incubated further for 1h at 4 °C. The plates were then washed, air dried and stained with 50 µl of 0.4% SRB dye in 1% acetic acid, followed by incubation for 30 min at room temperature. The unbound dye was then removed by washing with 1% acetic acid and kept overnight for drying. In each well, 100 µl of 10 mM tris-base was added to solubilise the dye followed by stirring for 5 min

at room temperature. The optical density was read at 570 nm using ELISA reader. The experiments were done in triplicates. Percentage cell growth was calculated as: Percentage cell viability = $[At/Ac] \times 100$
Percentage cell growth inhibition = $(100 - \text{percentage cell viability})$
Where At and Ac are absorbance of treated and control cells, respectively.

2.11. DNA content and cell cycle phase distribution

Human pancreatic (MiaPaca-2) cells were seeded in 6-well culture plates with cell density 2x10⁵ cells/ml/well and incubated for 24 hrs. After incubation, the cells were treated with PDEE (0, 30, 50 and 100 mg/ml) and again incubated for 48 hrs. After 48 hrs treatment cells were collected by 5 min centrifugation at 1000 rpm. The harvested cells were washed twice with phosphate buffer solution and fixed with 70 % ethanol at -20 °C for 1h. The cells were then stained with DNA staining solution containing propidium iodide (20 mg/ml) and triton X-100 (1%) in PBS for 30 min in dark. FACScan was used to measure DNA content. For each data file, data was collected from 10,000 cells. Cell Quest (Becton, USA) was used for analysis of histograms.

2.12. Loss of Mitochondrial Membrane Potential ($\Delta\Psi_m$)

Flow cytometry was used to measure the mitochondrial membrane potential loss ($\Delta\Psi_m$). Human pancreatic (MiaPaca-2) cells were plated in 6-well cultural plates with cell density of 1x10⁶ cells/ml/well and incubated for 24 hrs at 5 % CO₂ level. The cells were then treated with PDEE (0, 30, 50 and 100 mg/ml) and again incubated for 48 hrs. Rhodamine-123, a cell permeable cationic dye was added one hour before termination of experiment and again incubated for 30 min. The cells were washed with PBS and pellets were collected by centrifugation. The collected pellets were re-suspended in 300 ml of PBS. Florescence of Rh-123 in cells

were analysed at 485 nm by flow cytometer (Jung *et al.*, 2006).

2.13. Statistical analysis

All of the experiments were done in triplicate. The data were recorded as means $\pm$ standard deviations and were analysed with SPSS software.

3. Results and discussion

3.1. Total phenolic and flavonoid content

Polyphenolic compounds are very important plant bioactive constituents because of their scavenging potential due to the presence of hydroxyl groups (Hatano *et al.*, 1989). Folin-Ciocalteu method is most widely used to measure the polyphenol contents, with the basic mechanism of electron transfer and reducing ability (Prior and Schaich, 2005). Using this quantitative assay, we found that the total phenolic content (TPC) of ethanolic leaf extract of *P. denticulate* was found to be 12.24 ± 2.11 (mg GAE/g dry extract) as depicted in Figure 1. Considerable attention has been received by polyphenols for their physiological role as antioxidant and anticancer agents (Othman *et al.*, 2007). Polyphenolic compounds h-

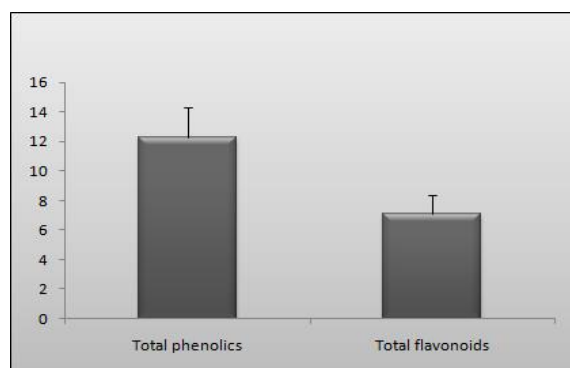


Figure 1: Represents the total phenolic and flavonoid content of PDEE. Each value represents the mean $\pm$ SD ($n = 3$).

-ave been reported to possess strong antioxidant potential their by scavenging free radicals and protect cells against such oxidative damages (Kahkonen *et al.*, 1999). The antioxidant potential of medicinal plants is due to the redox properties of polyphenolic compounds, which enables

them to act as strong reducing agents, hydrogen donor's metal chelaters and singlet oxygen quenchers (Miguel, 2010). The total flavonoid content in DPEE was found to be 7.06 ± 1.31 (mg catechin/g dry extract). Flavonoids are known exhibit many biological activities like antioxidant, anticancer, antimicrobial and anti-inflammatory properties (Hodek *et al.*, 2002).

3.2. DPPH free radical scavenging activity

DPPH is a purple stable free radical at room temperature with characteristic absorbance at 517 nm. The nitrogen free radical of DPPH is easily quenched by an antioxidant to yellow coloured complex (1,1-diphenyl-2-picrylhydrazine). The decolourization of purple colour is stoichiometric depending on the number of electrons gained (Soares *et al.*, 1997; Mokbel *et al.*, 2006; Singh *et al.*, 2002). DPPH radical scavenging potential of PDEE at different concentrations investigated in the present study was determined together with standard antioxidants (BHT and α -tocopherol) at the same concentrations (Figure 2). PDEE showed significant scavenging effect on DPPH free

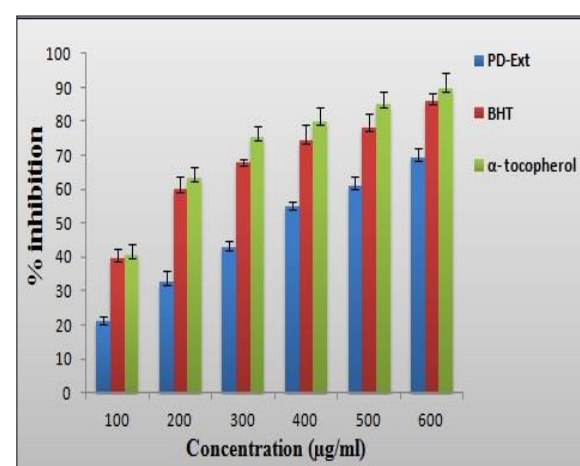


Figure 2: DPPH radical scavenging activity of PDEE and known antioxidant BHT and α -tocopherol. Values are means of triplicate experiments ($n = 3$) $\pm$ standard deviation.

radical in concentration dependent manner. When compared with standard anti-

oxidants used in the experiment, the extract showed relatively lower DPPH free radical scavenging potential. The extract at 600 µg/ml produced 69.35% inhibition, while as BHT and α -tocopherol produced 85.95% and 89.65% inhibition at the same concentration. The DPPH radical scavenging activity of PDEE as such might prevent reactive radical species from damaging biomolecules such as DNA, protein, polyunsaturated fatty acids (PUFA) and sugars in susceptible biological and food systems.

3.3. Hydroxyl ($HO^\bullet$) radical scavenging activity

Among different free radicals generated in biological systems, hydroxyl radical ($HO^\bullet$) is one of the most reactive species, which is capable to damage almost all the molecule in living cells, which ultimately leads to carcinogenesis and mutagenesis (Manian *et al.*, 2008; Hochstein and Atallah, 1988). This radical is considered to be one of the important initiators in the process of lipid peroxidation, abstracting hydrogen atoms from unsaturated fatty acids (Kappus *et al.*, 1991). In the present study, hydroxyl radical scavenging ability was estimated by generating hydroxyl radicals using ascorbic acid-iron- H_2O_2 (Fenton reaction). Antioxidant efficiency of PDEE was determined as the ability to scavenge the free radicals generated. The extract exhibited a concentration dependent scavenging of hydroxyl radicals which was comparable to the reference standards (BHT & α -tocopherol) at the same concentration. The percentage inhibition of hydroxyl radical scavenging is shown in Figure 3. The extract showed antioxidant activity in concentration dependent manner. A 120 µg/ml of PDEE, BHT and α -tocopherol exhibited 54.51%, 81.20% and 88.25% inhibition, respectively.

3.4. Prevention of oxidative DNA damage

Oxidative damage by hydroxyl radicals make DNA susceptible by oxidation of guanosine or thymine to 8-

hydroxyl-2-deoxyguanosine and thymine glycol which leads to mutagenesis and carcinogenesis (Ames *et al.*, 1993). PDEE prevents calf thymus DNA from oxidative damage due to hydroxyl radicals generated by $FeSO_4$ and H_2O_2 in Fenton reaction using agarose gel electrophoresis. Figure 4 shows the protective effect of PDEE on calf thymus DNA. The Hydroxyl radicals induce DNA strand breaks and causes complete DNA damage (Lane 2). PDEE at different concentration (10–100 µg/ml) offered concentration dependent protection to DNA damage (Lane 3-7). Catechin (10µg/ml) was used as standard antioxidant (Lane 8). Thus, the results indicate that PDEE prevents DNA damage against oxidative stress. The hydroxyl radical quenching ability of polyphenolic compounds of *P. denticulate* could be responsible for the protection against oxidative damage.

3.5. Anticancer activity

The anticancer activity of PDEE was determined by SRB assay against six human cancer cell lines. The assay is based on measuring the content of cellular protein using SRB dye (Vanicha and Kanyawim, 2006). Treatment of cells with PDEE (10–100 µg/ml) exhibited concentration dependent anti-proliferative effect (Table 1). The results of the present study reveal that the plant extract is very active against all the cell lines used. The highest percentage cell growth inhibition was observed in MiaPaca-2, HCT-116 and THP-1 with mean percentage value of 99.06 ± 0.30 , 98.45 ± 0.65 and 98.33 ± 1.25 respectively. The susceptibility of cells to the drug exposure was characterized by its IC_{50} values. Lower IC_{50} value indicates the higher anticancer potential of plant extract. The results of our study reveal that PDEE inhibits proliferation of cancer cell lines. The cytotoxic activity may be due to individual polyphenolic phytochemicals that act synergistically with other compounds to display the anticancer activity, as has been suggested by Yang *et al.* (2009).

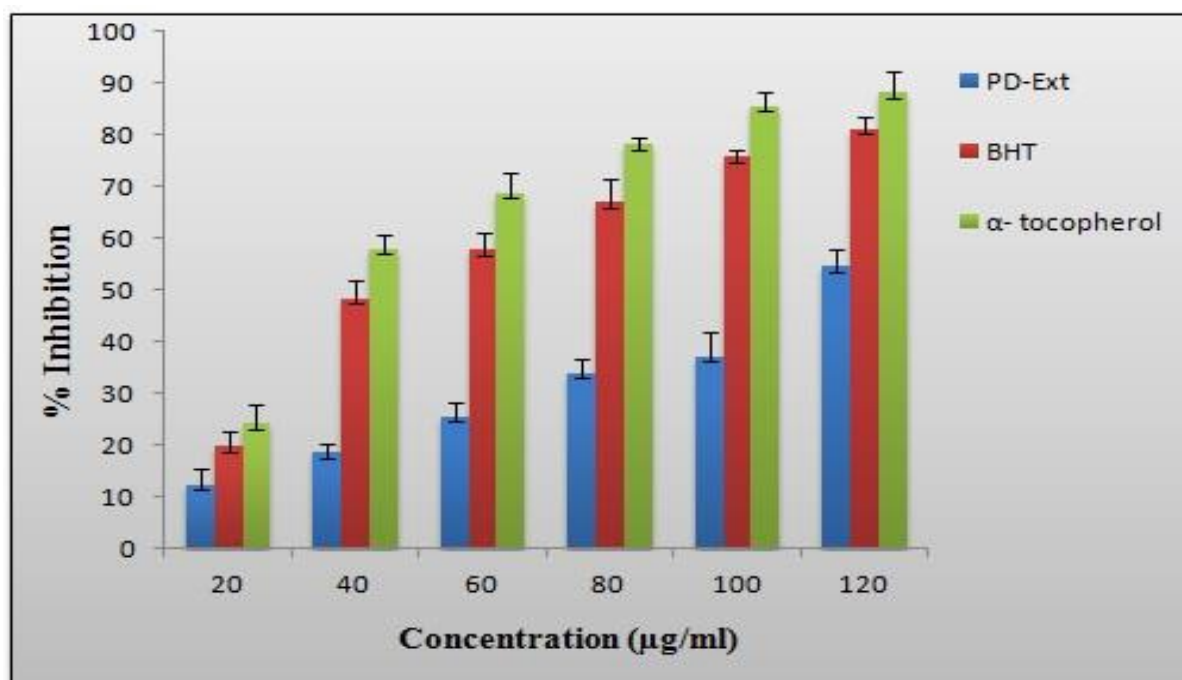


Figure 3: Effect of PDEE and known antioxidant BHT and α -tocopherol on hydroxyl radical scavenging potential. Values are means of triplicate experiments ($n = 3$) $\pm$ standard deviation.

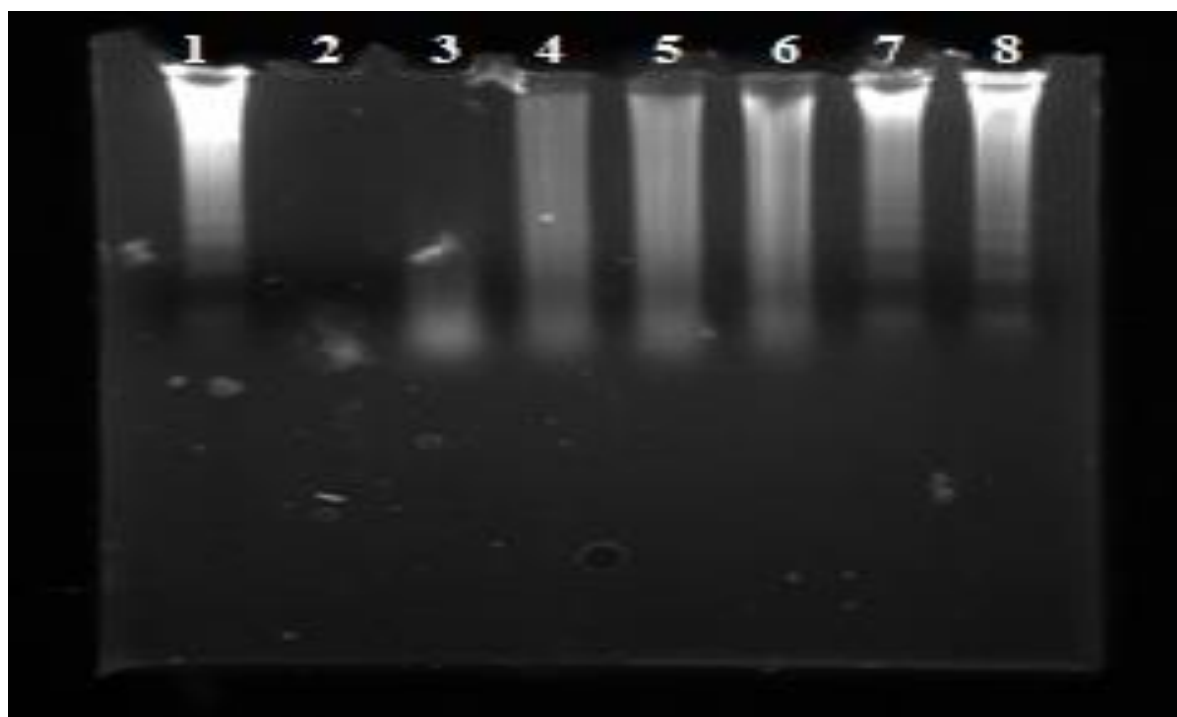


Figure 4: PDEE Protects calf thymus DNA from oxidative damage. *Lane 1:* Native calf thymus DNA; *Lane 2:* DNA + 20mM Ferric Nitrate + 100mM Ascorbic Acid + 30mM H_2O_2 ; *Lane 3:* DNA + 20mM Ferric Nitrate + 100mM Ascorbic Acid + 30mM H_2O_2 + 10μg of extract; *Lane 4:* DNA + 20mM Ferric Nitrate + 100mM Ascorbic Acid + 30mM H_2O_2 + 30μg of extract; *Lane 5:* DNA + 20mM Ferric Nitrate + 100mM Ascorbic Acid + 30mM H_2O_2 + 50μg of extract; *Lane 6:* DNA + 20mM Ferric Nitrate + 100mM Ascorbic Acid + 30mM H_2O_2 + 80μg of extract; *Lane 7:* DNA + 20mM Ferric Nitrate + 100mM Ascorbic Acid + 30mM H_2O_2 + 100μg of extract; *Lane 8:* DNA + 20mM Ferric Nitrate + 100mM Ascorbic Acid + 30mM H_2O_2 + 10μg of catechin.

Table 1: Anticancer activity of ethanolic leaf extract of *Primula denticulate*[#]

Sample	Conc. (µg/ml)	Percentage cell growth inhibition					
		MiaPaca-2 (Pancreatic)	A-549 (Lung)	PC-3 (Prostate)	THP-1 (Leukaemia)	HCT-116 (Colon)	HOP-62 (Lung)
PDEE	100	99.06 ± 0.30	95.67 ± 1.85	97.09 ± 2.09	98.33 ± 1.25	98.45 ± 0.65	93.68 ± 3.20
	50	73.15 ± 2.76	39.18 ± 1.90	40.23 ± 3.45	93.27 ± 2.10	83.21 ± 1.67	47.08 ± 2.34
	10	44.36 ± 2.09	17.05 ± 1.16	15.68 ± 2.53	38.35 ± 2.43	28.80 ± 1.56	10.96 ± 3.05
5-FU	20µM	-	-	-	67.87 ± 1.56	67.00 ± 1.87	-
Paclitaxel	1µM	-	70.78 ± 2.45	-	-	-	72.43 ± 2.78

[#]The results represent mean ± S.D of three experiments.

3.6. Flow cytometric analysis

To evaluate the action mechanism of cell growth inhibition by PDEE, further experiments were performed on human pancreatic (MiaPaca-2) cell line. In the present study, apoptotic induction was assessed by two assays; Cell-cycle phase distribution via propidium iodide (PI) staining flow cytometry and mitochondri-

al membrane potential loss using Rhodamine-123 staining flow cytometry. MiaPaca-2 cells stained with PI were treated with PDEE (0, 30, 50 and 100 µg/ml) for 48 hrs showed that the percentage of apoptotic nuclei increased to 57.00% at 100 µg/ml PDEE from 11.4% in control (Figure 5).

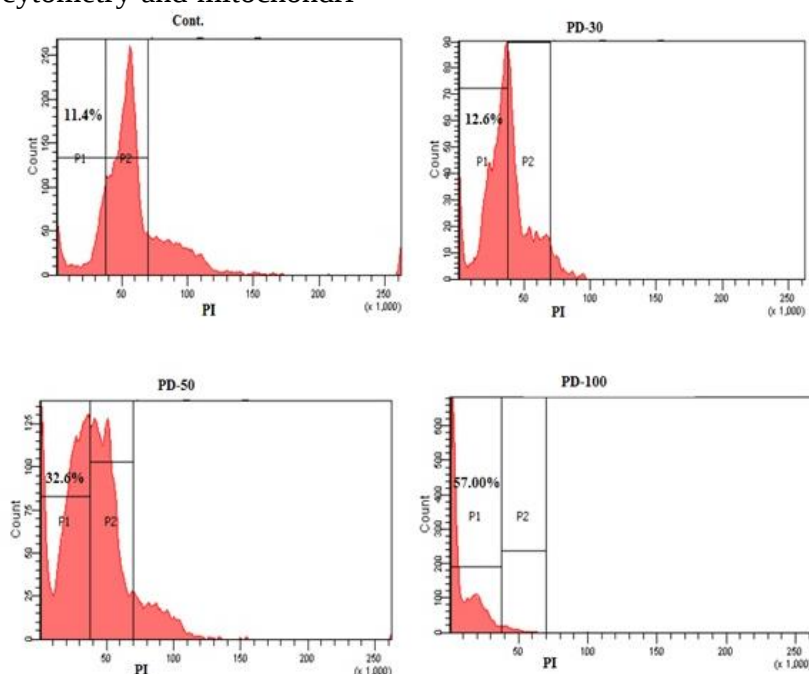


Figure 5: PDEE induces apoptosis of human pancreatic cancer (MiaPaca-2) cells via cell cycle arrest. Flow cytometric analysis of MiaPaca-2 cells after propidium iodide staining. Cells were incubated for 48 h in presence of PDEE (0, 30, 50 and 100 µg/ml). Figures show the representative staining profile of one of two similar experiments. P1 is the population of apoptotic cells, which increases from 11.4% in case of control to 57.00% in case of 100 µg/ml of PDEE.

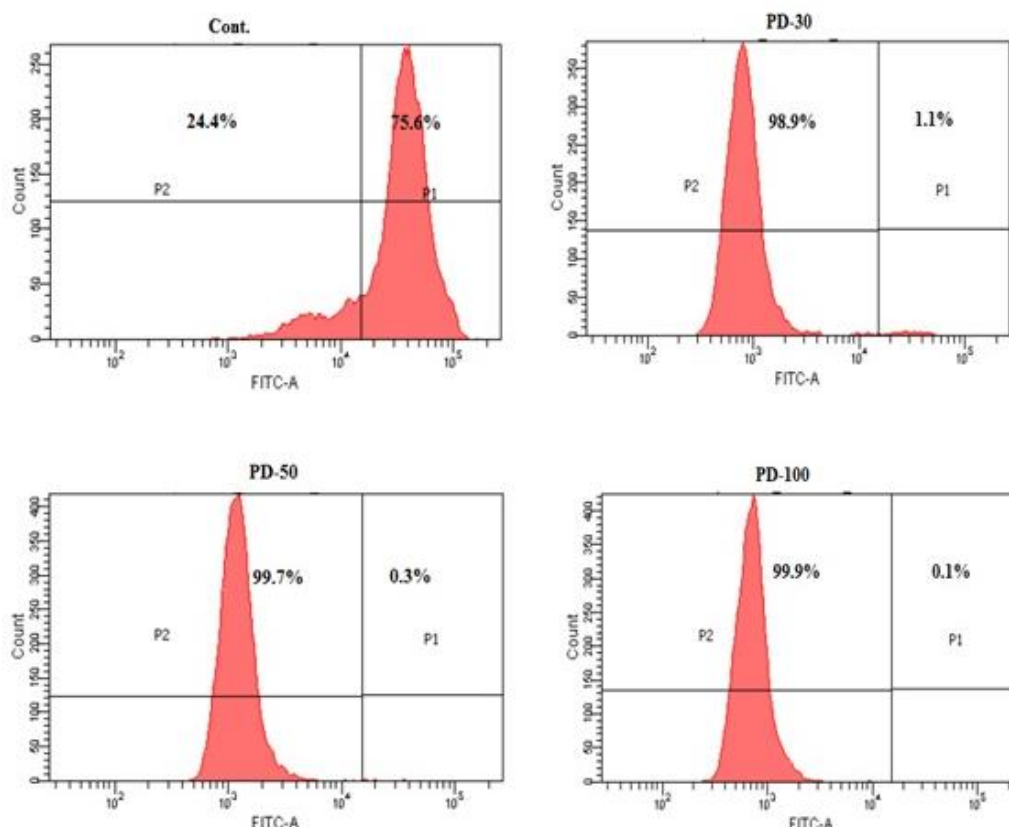


Figure 6: PDEE induced loss of mitochondrial membrane potential ($\Delta\Psi_m$) in human pancreatic cancer cell line (MiaPaca-2) incubated with extract at different concentrations (0, 30 50 and 100 $\mu\text{g/ml}$) in 6 well plate for 48 h treatment.

Apoptotic cells were characterised by degraded chromatin with high side-scatter (SSC) and low forward-scatter (FSC) properties (Bachir *et al.*, 2012). The results indicate that PDEE blocks cell cycle progression, resulted in significant increase in population of cells in sub G_0/G_1 phase, which may be due to fragmentation of DNA, resulted in apoptotic cell death. Disruption of MMP ($\Delta\Psi_m$) is one of the earliest events that occur following apoptosis induction (Qi *et al.*, 2010). MiaPaca-2 cells after treated with PDEE for 48 hrs resulted in loss of ($\Delta\Psi_m$) from 24.4% in control cells to a low of 99.5% at 100 $\mu\text{g/ml}$ of PDEE (Figure 6). MMP loss is mainly due to mitochondrial permeability transition pore, which causes Cytochrome-C release from mitochondria and ultimately triggers other apoptotic factors (Kroemer *et al.*, 1997). The increase in inner mitochondrial membrane permeability is due to interaction of anticancer agents with mitochondria (Fulda *et al.*, 1998).

4. Conclusion

The *P. denticulata* ethanol extract exhibit potent antioxidant property as revealed by different antioxidant assays. PDEE showed strong anticancer activity and induces apoptosis in MiaPaca-2 cell line by arresting cells at G_0/G_1 phase and inducing loss of MMP ($\Delta\Psi_m$). Further research is required to explore the potential of *P. denticulata* in developing an anti-cancer drug.

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Panchakavya: Organic Fertilizer and Its Stimulatory Effect on the Seed Germination of *Abelmoschus esculentus* and *Solanum melongena*

V. Ramya and S. Karpagam*

Department of Botany, Queen Mary's College, Chennai - 4, Tamil Nadu, India;

*Correspondence: s.karpagam98@gmail.com; Tel.: +91 9444944835

Abstract: Panchakavya is an incredible source of growth promoting substances. From ancient period cow's urine has been used as a medicine. In India, drinking of cow urine has been practiced for thousands of years. Panchakavya is a term used in Ayurveda to describe five important major substances, obtained from cow, which include cow's urine, milk, ghee, curd and dung all the five products possess medicinal properties against many disorders and are used for the medicinal purpose singly or in combination with some other herbs. This kind of treatment is called 'panchakavya therapy' or 'cowpathy'. The indiscriminate use of chemical pesticides resulted in environmental problems. An alternative to the chemicals is the natural products, one such as the panchakavya. Panchakavya is the single organic input that acts as a fertilizer, pesticide, growth promoter and immunity booster. The effect of panchakavya (cow urine) on the seed germination was studied in two plants namely *Abelmoschus esculentus* L.Moench (Lady's finger) and *Solanum melongena* L. (brinjal). The germination percentage was calculated. After germination the shoot and root length was measured and seedling vigour index was calculated. Cytotoxic effect of panchakavya on cell growth and cell division was studied in onion bulbs. The panchakavya at 1% concentration favoured the production of larger number of roots. This article highlights that panchakavya is more effective, easy to prepare, environmental friendly and could be used as a good fertilizer to boost the growth and productivity of agricultural crops.

Keywords: *Abelmoschus*: mitotic index; panchakavya: *Solanum melongena*: seed germination

1. Introduction

Agriculture is means of livelihood for millions of people in India and worldwide with crops chiefly dependent on rainfall and fertilizers. India is not only self-sufficient in food production but also has a substantial reserve (Gupta and Gopal, 2001). The latest trend is turned to organic farming, since the side effects of chemical fertilizers and pesticides have been established. India is the third largest producer and consumer of chemical fertilizers in the world. Heavy use of chemicals in agriculture has weakened the ecological balance, in addition to the degra-

dation of soil; water resources and quality of the food. At this juncture, a keen awareness has sprung on the adoption of organic farming as a remedy to cure the ills of modern chemical agricultural practice. It is very much essential to develop a strong workable and compatible package of nutrient management through organic resources for various crops, based on scientific facts, local conditions and economic viability (Nene, 1994; 1999). Cow is described as "Kamdhenu" (one which fulfills all the wishes) since vedic times in Indian civilization. According to ayurveda cow products are used to treat various disease conditions in human beings. Five

products of cow called as panchakavya is an important component of many rituals and pooja in Hindus. (Gosavi and Jhon, 2012; Sathasivam *et al.*, 2010). In Sanskrit, panchakavya means the blend of five products obtained from cow (all these five products are individually called 'Gavya' and collectively termed as 'Panchakavya'). When suitably mixed and used, has positive influence on living organisms. Panchakavya had got reverence in the scripts of Vedas (divine scripts of Indian wisdom), and Vrکشayurveda (vrکشha means plant and ayurveda means health system). The texts on Vrکشayurveda are systematization of the practices that the farmers followed at field level, placed in a theoretical framework and it defined certain plant growth stimulants; among them panchakavya was an important one that enhanced the biological efficiency of crop plants and the quality of fruits and vegetables (Natarajan, 2002).

Green revolution lead to intensified agriculture to meet the ever increasing demand for food and fiber, which is a practice at great cost to the environment resulting in continuous loss of natural ecosystems, ground water depletion, pollution and other environmental degradation (Gupta and Gopal, 2001). Alternative approaches to pest control is the concept of integrated pest management, where synthetic pesticides are only applied as a last resort and is now considered common practice in professional agriculture. The non-chemical alternatives include cultural practices, use of resistant varieties, creation of an environment favourable for natural enemies of pests and use of biological products and agents, including beneficial insects.

The indiscriminate use of chemical pesticides in modern agriculture resulted in the development of several problems such as pesticide resistant insects, resurgences of target and non-target pest, destruction of beneficial organism like honey bee, pollinators, parasites and predators and pesticide residues in food and fodder. The awareness about the

health and environmental problems due to the continuous use of pesticides resulted in the development of integrated pest management and organic farming.

Organic manure replaced chemical fertilizers, herbal extracts replaced pesticides and fungicides, but nothing was available to replace growth promoting hormones and immunity for plants. The organic system was imperfect and continued to be incomplete for want of an input to replace growth promoting hormones and immunity boosters, to maximize the efficiency of cultivated crops and coordinate the process leading to sustained higher productivity.

Many alternatives are available to reduce the effects pesticides have on the environment. Alternatives include manual removal, applying heat, covering weeds with plastic, placing traps and lures, removing pest breeding sites, maintaining healthy soils that breed healthy, more resistant plants, cropping native species that are naturally more resistant to native pests and supporting biocontrol agents such as birds and other pest predators.

Biological controls such as resistant plant varieties and the use of pheromones, have been successful and at times permanently resolve a pest problem. Integrated Pest Management (IPM) employs chemical use only when other alternatives are ineffective. IPM causes less harm to humans and the environment. The focus is broader than on a specific pest, considering a range of pest control alternatives. The use of phosphorous and nitrogen fertilizer in the global level has increased manifold, which effects the environment as run off into water bodies. The indiscriminate use of pesticide and fungicide has increased at global level, which is biomagnified at the tertiary level consumers.

Panchagavya or panchakavyam is a concoction prepared by mixing five products of cow and used in traditional Indian rituals. The three direct constituents are cow dung, urine, and milk; the two derived products are curd and ghee.

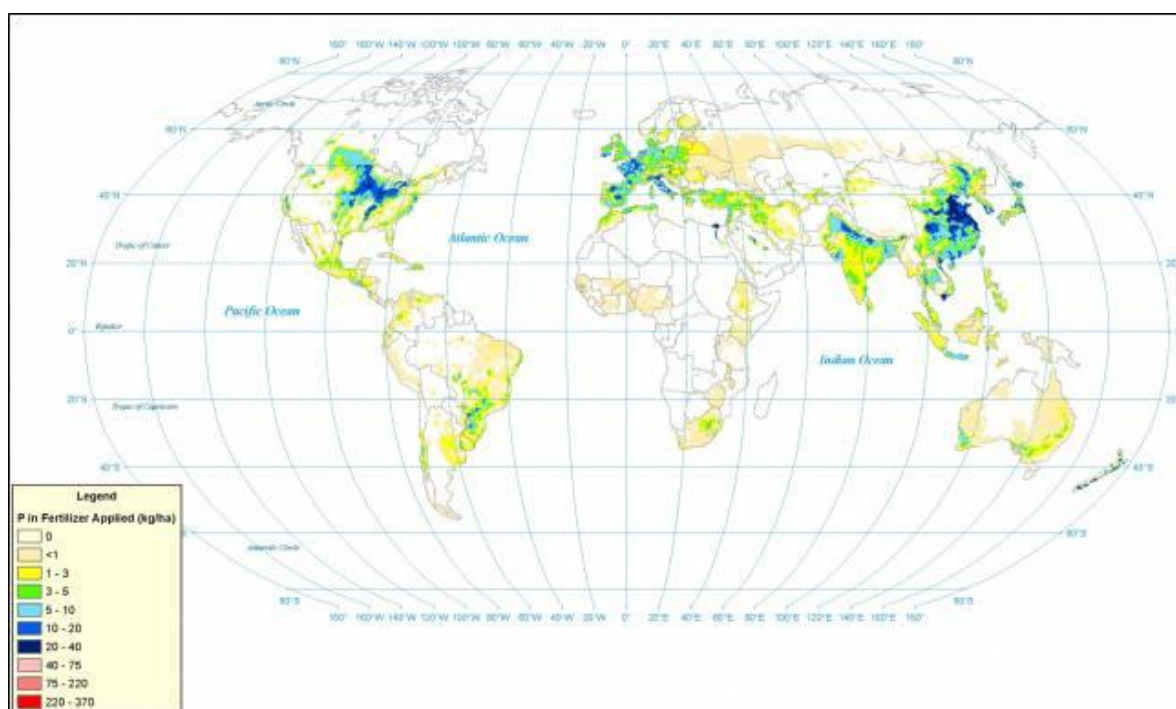


Figure 1: Global map of phosphorus fertilizer application rates (kg per ha of grid cell area) [source: <https://ourworldindata.org/fertilizer-and-pesticides/#note-6>].

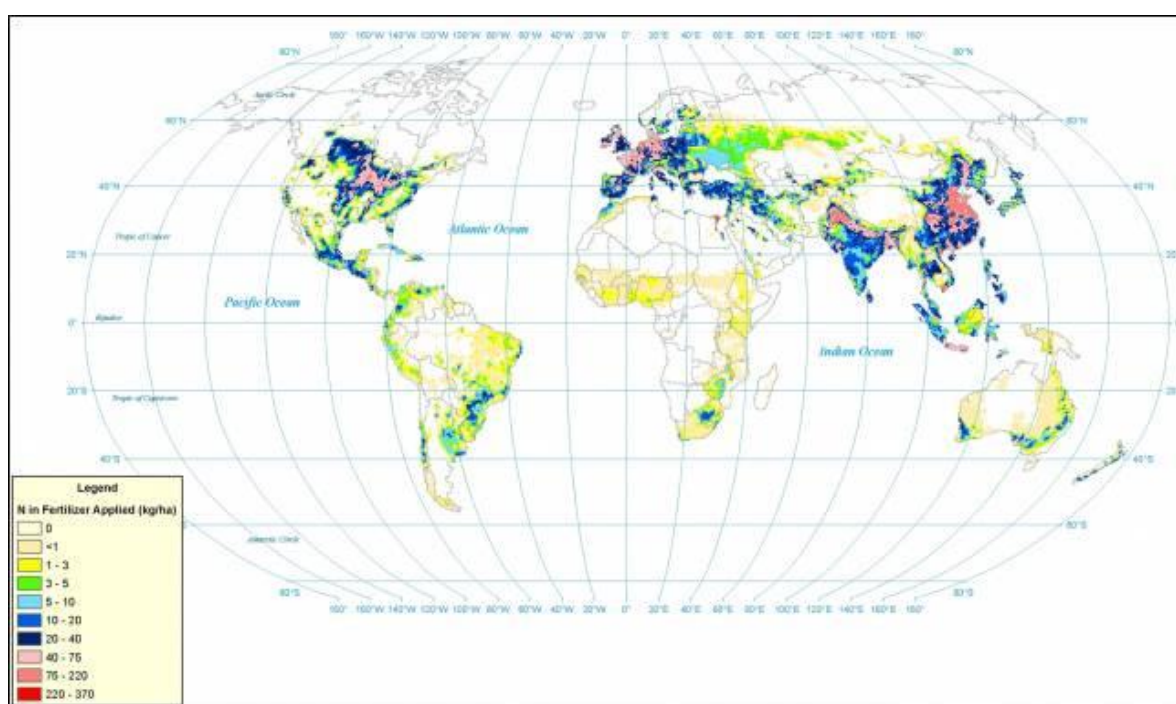


Figure 2: Global map of nitrogen fertilizer application rates (kg per ha of grid cell area) [Source: <https://ourworldindata.org/fertilizer-and-pesticides/#note-7>].

These are mixed in proper ratio and then allowed to ferment. Panchamrita is a similar mixture that replaces dung and urine with honey and sugar. The mixture which is made using yeast as a fermenter, bananas, groundnut cake, and the water of

tender coconut, is believed to be a potent organic pesticide and growth promoter - this is considered to be pseudoscience.

The Sanskrit word Panchagavya means "mixture of five cow products". It is

also called cowpathy treatment based on products obtained from cows used in Ayurvedic medicine and of religious significance for Hindus. Panchgavya is also used as fertilizers and pesticides in agricultural operations, but has no scientific evidence to back its claims.

Cow dung contained undigested fibre, epithelial cells, pigments and salts, rich in nitrogen, phosphorous, potassium, sulphur, micronutrients, intestinal bacteria and mucous, cow dung is also rich in bacteria, fungi and other microbial organisms (Nene, 1999). Singh (1996) recorded that cow dung had water 82% and solid matter 18%. Reddy (1998) reported that cows urine is rich in urea and acted both as nutrient as well as hormone. Cow milk was used by farmers in ancient times and reported to be an excellent sticker, spreader, a good medium for saprophytic bacteria and a virus inhibitor (Nene, 1999).

They are used as a Prasad in temples. A common usage is as a fertilizer and pesticide. Seeds can be treated with panchagavya. This was found useful in rhizome of turmeric, ginger and sugarcane and they yielded more, helps in plant growth and immunity. The medicinal usage of panchagavya, particularly cow urine, is practiced in Ayurveda. Proponents claim that cow urine therapy is capable of curing several diseases, including certain types of cancer, although these claims have no scientific backing. In fact, studies concerning ingesting individual components of Panchagavya, such as cow urine, have shown no positive benefit, and significant side effects, including convulsion, depressed respiration, and death. Cow's urine can also be a source of harmful bacteria and infectious diseases, including leptospirosis. Proponents claim it is an antibiotic growth promoter in the broiler diet, capable of increasing the growth of plankton for fish feed, the production of milk in cows, the weight of pigs, and the egg laying capacity of poultry chicken.

2. Materials and methods

2.1. Preparation of panchakavya

The ingredients for panchakavya sample was collected from cow farm (Thiruvallur DT)) using sterile container. Based on the detailed review of literature panchakavya stock solution was prepared by using cow dung (2.5 Kg), cow's urine (1.5 L), cow's milk (1L), cow's curd (1 L) and cow's ghee (0.5 kg). In addition, jaggery (1.5 Kg), tender coconut water (1.5 L) and ripe banana (6 Nos.) were also added as modification. All the materials were placed in a wide mouthed mud pot and kept open under shade. The contents were stirred twice a day for about 20 minutes, both in the morning and evening to facilitate aerobic microbial activity. The panchakavya stock solution will be ready after 30 d (care should be taken not to mix buffalo products, local breeds of cow is said to have potency then exotic breeds) and is covered with a plastic mosquito net to prevent houseflies from laying eggs and the formation of maggots in the solution. Jaggery is dissolved in water and used while sugarcane juice is more suitable.

2.2. Seed collection

The seeds of *Abelmoschus esculentus* (ladies finger) and *Solanum melongina* (Brinjal) were bought from nursery Balaji traders, Chennai 601203, Tamil Nadu. Healthy Seeds of uniform size and shape were used for sowing.

2.3. Treatments

Treatments were given as: 1. Control (water); 2. Chemical (Cartap hydrochloride 50% radon sp + tata tafgor dimethoate 30% ec = 2:1 ratio and dissolved in 10 ml of water); 3. Panchakavya; 4. Panchakavya +Neem cake; 5. vermicompost were used.

2.4. Germination of seeds

The seeds (40 numbers) were soaked in sterile water in Petri plates with filter paper. After 24 hours, the seeds were observed for germination. The ger-

minated seeds were counted, the radical and plumule length were measured (cm) and tabulated.

2.5. Seedling length

Two days after seed germination, ten normal seedlings were taken out carefully at random from each treatment and seedling length was measured from the tip of primary root to the tip of apical shoot. The average length (cm) of ten seedlings was calculated and expressed as mean seedling length.

2.6. Seedling vigour index

The seedling vigour index was calculated adopting the method suggested by Abdul-Baki and Anderson (1973) and expressed whole number treatment wise

$$\text{Vigour index} = \frac{\text{Germination percentage}}{\text{Seedling length}}$$

2.7. Effect of panchakavya on cell division and cell growth

Cytological studies of root tip of *Allium cepa* was studied for 7 days. Onion bulbs were treated with diluted panchakavya (50%, 25%, 10% and 1%) and tap water as control. *Allium cepa* (2n=16) (Rank and Nielsen 1994) were used as test system. The root tip from control and experimental setup were thoroughly washed in distilled water and fixed in Carnoy's fixative (ethanol, chloroform glacial acetic acid 6:3:1 v/v/v) and chromosome studies were done by acetocarmine staining technique. The fixed root tip were washed in distilled water and hydrolyzed in 1N HCL for 10 min then treated in 45% acetic acid for 5 min and stained in acetocarmine stain for 10-15 minutes. After staining the root tips were squashed and observed under phase contrast microscope.

3. Results and discussion

The effect of panchakavya on seed germination of two plants namely *Abelmoschus esculentus* and *Solanum*

melongena was studied in sterile Petri plates. Seeds of 40 numbers were soaked in 2ml of water (Control), in 2ml of Chemical control, (Cartap hydrochloride 50% radon sp + tata tafgor dimethoate 30% ec = 2:1 ratio and dissolved in 10 ml of water), and 2 ml of panchakavya; 2ml of Panchakavya + neem cake (2 gm), 2ml of vermicompost as treatment, separately and was observed for 5 days. The germinated seeds were counted and the germination percentage was calculated in each of the treatment (Table 1 and 2). The germination frequency was 100% in panchakavya and the adjuvant treatment in *A. esculentus* while it was only 90% in *S. melangena*. The germination frequency was lesser in all the other treatments and it was only 50% in control for *S. melangena*. Among the panchakavya treated seeds, maximum germination was found in both *A. esculentus* and *S. melongena*.

After germination the seedling vigour index was calculated by measuring the length of shoot and root. The data on shoot and root length of different treatment were measured by cm scale.

The plants treated with 2ml panchakavya produced the longest shoot length (2.3) and root length (2) in *Abelmoschus esculentus*. While in *Solanum melongena* longest shoot (1.2), root length (1.5) was in panchakavya treatment which was comparatively higher than control. The seedling vigour index was higher in panchakavya treated seedlings in both the plants which was 430 for *A. esculentus* and 243 for *S. melangena* which was higher than control and all other treatments (Table 1 and 2).

Among the panchakavya and panchakavya + neem cake treated seeds, maximum germination frequency was observed in *Abelmoschus esculentus* and *Solanum melongena*. The shoot length was 2.5 and 1.2 in *A. esculentus* and *S. melangena* and root length was 2 and 1.1 in *A. esculentus* and *S. melangena*. There was a slight difference in 2ml of panchakavya + neem cake. The pan-

chakavya and panchakavya + neem cake, is more effective, when compared to control as well as other treatments. The 100 % germination was observed in *A.esculentus* in panchakavya and panchakavya + neem cake and while only 90 % germination was seen in *Solanum melongena* (Table 1 and 2).

The growth of onion roots was observed on panchakavya at the concentrations of, (50%, 25%, 10%, and 1%). The highest number of onion roots, shoots length and root length was observed in 1 % concentration of panchakavya, and it

was measured in (cm) scale (Table 3 and 4).

The onion bulb developed more number of roots in 1% panchakavya which was 48, and the root length was 7.5, and the shoot length was 7 which was higher when compared to other concentrations. At 1% concentration of panchakavya the number of cells in the microscopical field was 250 cells, of which 48 of them was in metaphase stage. Whereas, in control it was lesser, 22 cells in metaphase stage out of the 250 cells in the microscopical field. The 1% concentration of panchakavya increased the

Table 1: Pesticide consumption (in lakh tones) during, 1994-1997 (Heisey and Norton, 2007)

Country/Region	Herbicides	Insecticides	Fungicides
China	NA	NA	NA
India	6.8	37.2	9.4
Other Asia	24.4	41	19.2
Middle East/North Africa	9.7	19.5	14.1
Sub-Saharan Africa	11.7	9.7	9
Latin America/Caribbean	85.8	39.8	31.8
All developing	138.4	147.2	83.6
Transitional	35.6	7.9	23.2
Industrialized	337.8	163.4	190.4
World	511.8	318.4	297.2

Table 2: Rate of seed germination in *Abelmoschus esculentus*

No	Treatment	Time Duration (Hours)	No of Seeds	No of seed germination	Germination %	Vigour index Seedling Length
1.	Control	48	40	28	70	98
2.	Chemical	48	40	32	80	184
3.	Panchakavya	48	40	40	100	430
4.	Panchakavya + Neem cake	48	40	40	100	450
5.	vermicompost	48	40	40	100	410

Table 3: Rate of seed germination in *Solanum melongena*

No	Treatment	Time Duration (Hours)	No of Seeds	No of seed germination	Germination %	Vigour index Seedling Length
1.	Control	48	40	20	50	70
2.	Chemical	48	40	28	70	133
3.	Panchakavya	48	40	36	90	243
4.	Panchakavya + Neem cake	48	40	36	90	207
5.	vermicompost	48	40	32	80	168

Table 4: Effect of Panchakavya on growth of onion roots

S.No	Concentration [%]	No. of roots After four days	Root length [cm]	Shoot length [cm]
1.	Control	26	5	3
2.	50	37	5.5	5
3.	25	37	6	6
4.	10	37	7	6.5
5.	1	48	7.5	7

number of onion roots, when compared with control as well as other different concentration of panchakavya.

The greatest challenge of the nation in the coming years is to provide safe food for the growing population in the country without degrading the environment. In this regard organic farming which is a holistic production management system for promoting and enhancing health of agro ecosystem, has gained wide recognition as a valid alternative to conventional food production and ensure safer food supply for human consumption. This farming system avoid large use of synthetic fertilizer, pesticides, growth regulators and livestock feed additives and relies on green manures, crop rotation, crop residues, animal manures, bio fertilizers, bio pesticides, different kinds of cow based liquid organic manures such as panchakavya, sanjibani, kunapajala, amrit pani etc.

Many advanced countries mainly depend upon the dairy products because of their commercial, agricultural and nutritive properties. The dairy industries play a vital role in the development of the country. When a new house or building or even a temple constructed in India, the first to enter the premises would be the cow because this is considered to be auspicious. Cow's urine (Comiyam) is used in almost all the Hindu rituals. The potential of using panchakavya as growth promoter and biofertilizer is revealed in this work. The present study revealed the germination frequency of *Abelmoschus esculentes*, and *Solanum melongena* grown under the different treatments namely, Control, Chemical + fertilizer,

panchakavya, adjuvated with neem cake, and vermicompost.

The effect of adjuvants namely vermicompost and neem cake showed a slight difference, whereas the panchakavya alone was sufficient to enhance the seed germination. The panchakavya favors cell growth, cell division (Table 5) which is known from the mitotic index. The onion bulbs produced longer roots and shoots when compare to control. The panchakavya at 1% concentration favoured the production of larger number of roots. The presence of growth promoting substance present in the panchakavya favored rooting. The present study reveals that panchakavya preparation is easy and it is cost effective and could be prepared easily by a farmer with his household expenses and availability. Rahul kumar *et al.* (2014) studied the effect of panchakavya fortified with *Bauhinia* plant extract which showed a positive response as an anthelmintic preparation.

Table 5: Effect of Panchakavya on cell division [onion root tips]

No	Concentration [%]	No. of cells in Metaphase	No. of cells
1	Control	22	250
2	50	46	250
3	25	44	250
4	10	45	250
5	1	48	250

Sangeetha and Thevanathan (2010) studied the potential of panchakavya as biofertilizer against certain pulses by growing in soil amended with seaweed extract and panchakavya. Experimental

seedling recorded higher rates of linear growth of both shoots and roots as compared to controls. These seedlings produced 264 to 390% more lateral roots than the control and maximum lateral root production was always observed in seedlings grown in soil amended with seaweed based panchagavya at low concentrations

In recent years the people have recognized a number of commercial, medicinal and agricultural values from the various products of dairy forms. Tharun *et al.*, (1983) carried out extensive works in this aspects and the environmental management in developing countries.

The number of new methods of recycling and controlling measures of organic waste in urban and rural habits was proposed by (Furedy *et al.*, 1989). The current global scenario firmly emphasizes the need to adopt eco-friendly agricultural practices for sustainable agriculture. Chemical input had an adverse impact on the health-care of not only soil but also the beneficial soil microbial communities and the crops. This eventually led to a high demand for organic products. Farmers all over the world realize the need to detoxify the land by switching over to organic farming dispensing with chemical fertilizers, pesticides, fungicides and herbicides. In India, organic farming was a well developed and systematized agricultural practice during the past and this ancient wisdom obtained through Indian knowledge is the use of 'panchagavya' in agriculture for the health of soil, plants and humans.

4. Conclusion

Panchakavya is used in different forms. Such as foliar spray, soil application along with irrigation, seed treatment or seedling treatment etc. For foliar spray 3% concentration is being used by organic farmers. Panchakavya was an important one that enhanced the biological efficiency of crop and the quality of fruits and vegetable production. Panchakavya not

only enhances the plant yield and growth rate, it also reduces the insect invasion and fungal attack. The results of the present study clearly show that panchakavya is an organic fertilizer and growth promoter. It also increases the soil fertility. It is very much essential to develop a strong workable and compatible package of nutrient management through organic resources for various crops, based on scientific facts, local conditions, and economic viability for the sustainability.

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Short Communication

Increasing Human Interference in Katarniaghat Wildlife Sanctuary

Shiv Pratap Singh*

*Department of Geography, Kisan P.G. College Bahraich, Affiliated to Dr. R.M.L. Awadh University, Faizabad, Uttar Pradesh, India; *Correspondence: principalkpgc@yahoo.co.in; Tel.: +919984146483*

Abstract: Katarniaghat Wildlife Sanctuary (KWS) is a part of Dudhwa Tiger Reserve. It is managed with the Dudhwa National Park and Kishanpur Wildlife Sanctuary. It is located near to the Indo-Nepal border. It is in the Bahraich district in Uttar Pradesh. The sanctuary comes under the tropical moist deciduous forest of the Himalayan Tarai-Bhabhar region. It is under laid on 31 May, 1976. It is situated between 27°41 – 27°56 N and 81°48 – 81°56 E covering an area of 551 km. The KWS is divided into six divisions. Four division (Katarniya, Nishangada, Murtiha, Dharampur) of it lies under the core zone and remaining two divisions are in buffer zone. Tharu is the main tribe of this area. Increasing human interference has been studied in the related research paper that outsteps the concept of Biosphere Reserve. Due to growing tourism and farming in core zone, and human activities and habitats in buffer zone, it has become a critical situation to the bio-diversity of this wildlife sanctuary. In this article, the challenges of the Katarniyaghat Wildlife Sanctuary are highlighted. The highlighted challenges need to be addressed for the sustainable growth and development of the region and the associated communities.

Keywords: Biodiversity; conservation; environment; human interference; sustainable development; wildlife

1. Introduction

In the 21st century, due to growth in materialistic view of life and population explosion, the competition to make the life more and more comfortable by utilizing industrial products has become noticeable. To meet the consumers demand, industrial production is increased drastically; but unplanned and indiscriminate industrialization elevated the level of environment pollution and ecological imbalance. The growing number of animals and human, and pressure of agriculture and economic development destroyed the forests. Deforestation is sensitive in India; because not only it has a bad effect on the

environmental activities but availability of fuel, food items, fodders and income also get affected. In this article, the example of Himalayan Tarai Ecosystem of Uttar Pradesh and the problems pervaded in biodiversity of Katarniyaghat Wildlife Division are highlighted.

2. Introduction of the study field

Katarniyaghat Wildlife division is situated in Nanpara Tahsil in Bahraich district of Uttar Pradesh in India. It lies along Indo-Nepal international border. It is situated between 27°41 - 27°56 N and 81°48 - 81°56 E. This division is extended in about 551 km area; which is an im-

portant part of Himalayan Tarai - Bhabhar region. This wildlife sanctuary is included in the Project Tiger in 2003 for the conservation of tigers, Saryu (Kaudiyala and Girwa) river Dolphins, massive Crocodiles, Alligator and Turtles biodiversity.

The forest of the sanctuary area has been classified into two major types: (1) The Sal forest and (2) the miscellaneous forest. This area is rich in biodiversity by including vast grasslands, dense forests of Sal, Sakhu and Teak trees and Aquatic areas.

Pedagogically, the study area is made up of the alluvial soil of the Saryu River and its tributaries flowing adjoining to it.

The study area has a tropical monsoon type climate with three distinct seasons i.e. summer (April to June) winter (November to February) and warm-rainy (July to September). March and October are considered as transition months between the seasons. The mean maximum temperature ranges from 22 degree Celsius in January to 40 degree Celsius in May and the mean minimum temperature ranges from 8 degree Celsius in January to 27 degree Celsius in June. The annual rainfall ranges from 36 to 142 cm in winter, 34 to 662 cm in summer and 1294 to 1689 cm in warm-rainy seasons.

It is a natural aesthesis to see roaring Tigers, Leopard resting on tree, leaping Cheetal, Padha, Stage, Kakad and Boars digging the forest land with their long snout.

To conserve the abundance of wild lives, U.P. government has divided it in 6 sub divisions. Four sub divisions (Katarnia, Nishangara, Murtiha, Bharthapur) are declared as a core zone and remaining two (Motipur, Kakraha) are declared as a buffer zone. North-east railway line passes through this division connecting the Bichhia-Katarniya tourist place. This line is parallel to the roadway that divides the zone into two parts (Figure 1).

There is greatest biodiversity under the Katarniya zone. If we have a glimpse on biodiversity of biosphere reserve then we find endangered Ganga Dolphin,

Tiger, Cheetal, Stages, Rhinoceros, Elephant, Leopard, Boar, Padha etc. Under the endangered aquatic birds we find Lalsar, Surkhab, Nilsar, Gugal, Kaj kurchhia and little Mew. Some other birds like Peacock, Moorcock, Pheasant, Dhanesh, Log log, Hairns, Submarine bird, Crane, Wilture, Hawak, Kite, Owl, Caprimulgid, Magpia, Woodpecker, Khanjan, Mynah bird, Crow, Nightangle, Satbahin are also observed.

Endangered reptiles namely, Iguanas, Pythans, Black Cobras and Cobras are found here. For conserving crocodiles and alligator a Crocodile Project has been established in the Katarniyaghat. Currently, this wild life division is being developed as a tourist place of Bahraich district. Tourists come here to see and watch wild animals and birds natural beauty as well from for-off places. The main tribe living here is '*Tharu*' which lives in the buffer zone of the forest area. They are able to earn their livelihood by working in agricultural or non-agricultural sectors.

3. Katarniyaghat wildlife sanctuary and the concept of biosphere reserve

Under the concept of bio-sphere reserve the conservation and enhancement of endangered environmental condition is implied. The entire zone is divided into three zones. First zone is the specific zone related to the internal part; where human entries are restricted. Second zone is known as transitional zone or mid-zone surrounded to the core zone; this zone is related to research centers, tourist places and utilization of fuels and non-living resources. Third zone which is situated round the mid zone is related to the habitats, agriculture, tourist places and utilization of non-living resources. But in the Bio-sphere reserve (Katarniyaghat Wildlife Sanctuary), the core zone (Katarnia, Nishangara, Murtiha, Bharthapur) is being developed as Guest House (GH) and Tourist Centre (TC). Meanwhile, in the buffer zone, the activities of forestry and agriculture are growing for the earning of

livelihood for growing population density. Thus, the area of this zone is decreasing day by day. If these trends persist then

there is possibility of bio-diversity loss and imbalance of ecosystem.

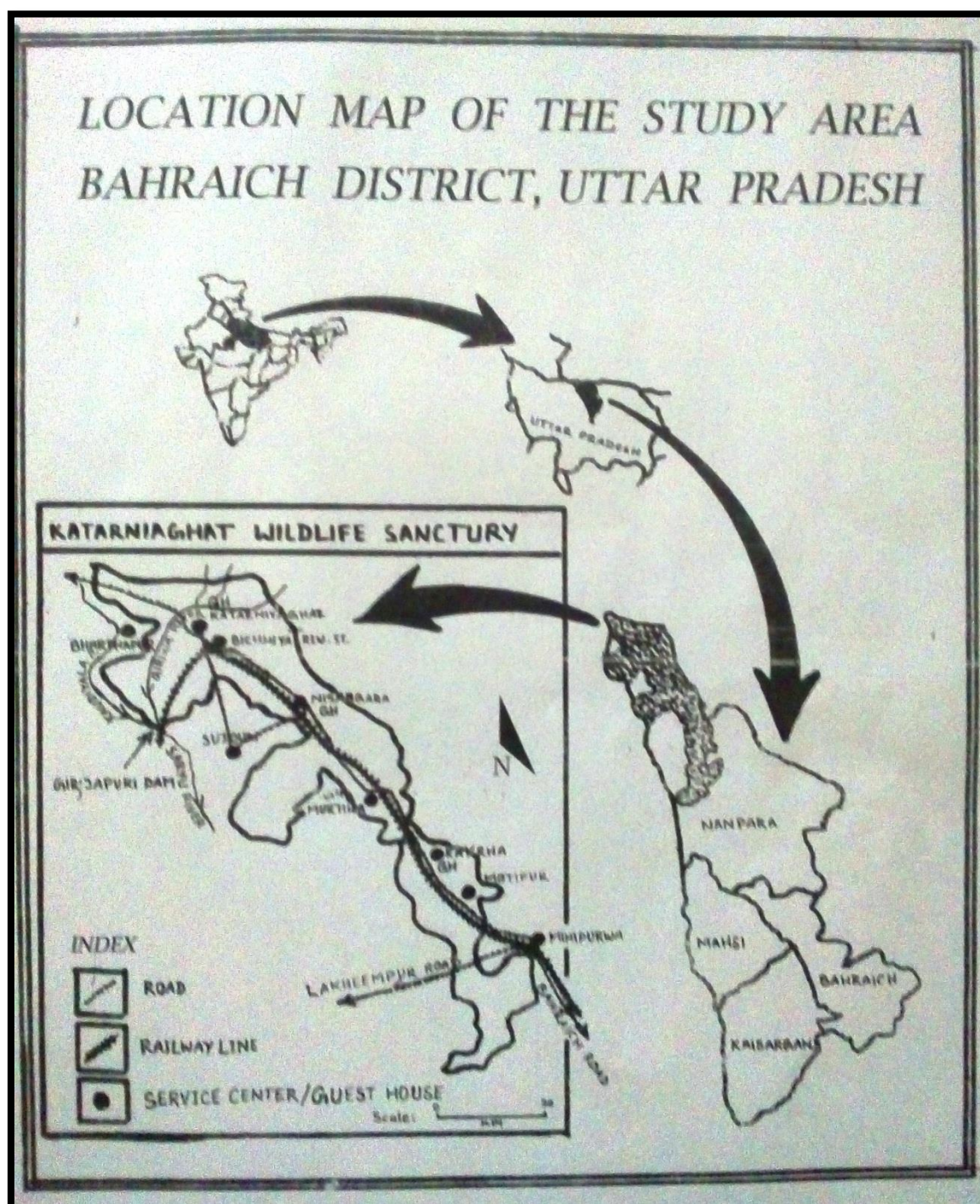


Figure 1: A diagrammatic sketch (map) showing the Katarniaghat Wildlife Sanctuary location in India. Katarniaghat Wildlife Sanctuary map shows the roads, railway lines and guest houses located within the sanctuary.

4. Human activities in Katarniaghat wildlife sanctuary

Approximately 20% of the area consisting mostly of the grassland is infested

by *Lantana sp.* Over grazing and human activities including fire are disturbing. The Protected area also experiences pressure for fuel wood and fodder from the adjoining 25 villages and also from popu-

lation on the Nepal side. There are some forest villages (erstwhile Taungya villages) and a Central State Seed Research Farm near Girijapuri, situated inside the wildlife sanctuary covering 38.42 sq Km area that causes considerable disturbance to the wildlife, creating a break in the forest connectivity.

Generally, we know that the harnessing of timber and fuel is right and to cut the forest for trade is illegal. It cannot be restricted without giving the employment to the effected people and maintaining facultative measures. In the studied area, the most of the human activities are done by rural people; because, the activities of cattle rearing and forestry are done directly. Along with these activities, illegal wood cutting and hunting is found in abundant measure. In the buffer zone of this sanctuary, the rural population and unplanned habitats are growing continuously. So the local people are overleaping the forest land and converting it into agricultural land by cutting the forest. In fact is a challenge of present time for the sanctuary. In addition to this, the fast running vehicles and north-east railway line cause the accident of wild lives of the forest area. The ecological system is widely disturbed by the noise pollution and savage pollution caused by the tourists. As the Sanctuary is situated at the border of Nepal; Nepalis from the border region are involved in lot of illegal activities related to forestry; which is also disturbing to the wildlife and forest of the Sanctuary.

5. Response to cope with the changing theme (human activity)

Though the management plant of the Protect Area Sanctuary (PAS) and working plant of Reserve Forest Sanctuary (RFS) have not been specifically oriented to cope with the identified Global Change Factors per se yet, Many initiatives are taken up to minimize the factors that contribute to habitat fragmentation and infestation of invasive species. Major factors

responsible for the fragmentation are; as follows:

- Burgeoning human population (and therefore the escalating pressure for resources)
- Changed socio-economic scenario (e.g. changed lifestyle of the Gujjars and Khatta holders, therefore overgrazing over cutting of firewood timber and fodder species)
- Encroachment of forest lands (by agriculture monoculture plantation and other land use)
- Infrastructure development (like rail, road, hydroelectric and irrigation projects)
- Various illegal/ legal (timber harvesting, boulder mining in river beds etc.)
- approach of various departments
- Little/ no community participation
- Obsolete policies less amenable to adapt to changes, and
- Lack of adequate scientific knowledge and proper monitoring plants.

The magnitude of the above stated problems leading to habitat fragmentation and biological invading varies. There are different responses from various stakeholders. The government forest department, local communities, and the main stakeholders that largely manage and use the natural resources of the landscape. There are some broad responses, which could be generalized across the sites for the betterment of the sanctuary.

6. Perspectives

This paper highlighted the human activities in which are disturbing wildlife sanctuary. To protect the biosphere reserve people should be educated to inculcate the importance of conserving wildlife for the sustainability. The human interference in the buffer and core zone generated the danger to the bio-diversity. Forest department has recently started the plans to supply income to the local people but it is well know that only forest department

cannot do this. Other institution will have to take responsibility to make more chances to generate employments for the people in the vicinity of the sanctuary. *Jawahar Rojgar Yojna, Samekit Gramin Vikas Karyakram* and *MGNAREGA* will be helpful in providing proper jobs to locals to establish the right situation to conserve the wild lives. Task is challenging; but, it is essential for the sustainable growth and development of the people and the region.

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**“Earth provides enough to satisfy every
man's needs, but not every man's greed”**

--- Mahatma Gandhi

About Editors

Subhash Bhore, PhD: Subhash completed his BSc (Botany) and MSc (Botany) degrees education at University of Pune, India. Immediately after completing his MSc (1996), he got an opportunity to work at 'Biochemical Engineering Department' and 'Plant Tissue Culture Pilot Plant' of the CSIR-National Chemical Laboratory, Pune, India. In June 2000, he received a Doctoral Fellowship (GRA) to pursue a PhD Degree in Molecular Genetics at the National University of Malaysia (UKM). In 2004, he was appointed as Senior Research Officer at Melaka Institute of Biotechnology (MIB), a research wing of Melaka Biotechnology Corporation, Malaysia. Based on his performance, in April 2005, he was promoted as 'Principal Investigator & Head of R&D Department' at MIB, Malaysia. In 2008, he was invited by the AIMST University as a 'Visiting Faculty' for their Department of Biotechnology and now serving as a Senior Associate Professor. In 2009, he was nominated for the AASIO (Association of Agricultural Scientists of Indian Origin) Young Scientist Award. He has published more than 50 peer-reviewed articles, 6 books and submitted more than 11,900 DNA sequences in Gene Bank; filed one patent, and received more than 10 awards/fellowships. As of June 2017, he has supervised more than 72 students including postgraduates, undergraduates and industrial trainees. He is actively involved in research as well as teaching and advising of postgraduate and undergraduate students. You may contact him using email, subhash@aimst.edu.my or subhashbhore@gmail.com



Kasi Marimuthu, PhD: Marimuthu accomplished his BSc (Zoology); MSc (Environmental Biotechnology); PhD (Environmental Biotechnology/Zoology Interdisciplinary) degree education at Manonmaniam Sundaranar University, Tamilnadu, India. In 2003 he joined as a Post-Doctoral Fellow at School of Biological Sciences, University Science Malaysia, Penang for 2 years. At present, he is working as a Professor in the Department of Biotechnology AIMST University, Malaysia for the last 12 years. He teaches Aquaculture, Biostatistics, Research Methodology, Biology of Invertebrates and Vertebrates courses for undergraduate biotechnology programme. His research interests include fish reproduction and breeding, larval rearing, hatchery management, fish immunology and aquatic toxicology related research. He has published 95 research papers in fisheries and aquaculture in various reputed and indexed journals. He has participated in more than 35 local and international conferences, seminars, and workshops. He has been appointed as an external examiner for six Indian Universities (Manonmaniam Sundaranar University, Annamalai University, Bharathiyar University, Bharathidasan University, Madras University, and Priest University) Tamilnadu, India. He is a life member in National Academy of Biological Sciences [NABS], India and Asian Fisheries Society. He is an editorial member in Indian Journal of Natural Products and Resources and Acta Ichthyologica Et Piscatoria. He is also presently serving as a Deputy Vice chancellor for Academic and International Affairs, AIMST University, Kedah Darul Aman, Malaysia. You may contact him at marimuthu@aimst.edu.my / aquamuthu2k@gmail.com.



Manickam Ravichandran, PhD: Prof M. Ravichandran obtained his M.Sc Medical Microbiology from Christian Medical College, Vellore in 1991 and gained PhD degree from Anna University, Chennai, India in 1997. Currently his research interests include cholera vaccine, molecular diagnostics and phage therapy. He has constructed several cholera vaccine candidates for O139 Vibrio cholerae and developed several

simple cold chain free molecular diagnostic kits. He has published 64 papers in international journals (Cumulative citations 584), supervised 40 postgraduate students, filed 8 patents and commercialized 3 products on diagnostics. He has been awarded with 44 national and international awards for the academic and research excellence including Ideas Inventions New Products (IENA), Nuremberg Germany; International Exhibition of Inventions: New Technologies and Products, Geneva and Anugerah Inovasi Negara. He is an associate member of Academy of Sciences Malaysia (ASM) and Expert panel member for R,D&C grants, Sciencefund, Technofund, Community Innovation Fund (CIF), Enterprise Innovation Fund (EIF), and Innovation (MOSTI); He was the Cluster Working Group (CWG) Committee member on Human Capital Development, Malaysian Biotechnology Corporation and the Committee member on 'Top Research Scientists Malaysia'(TRSM) of Academy of Sciences Malaysia (ASM), Biotechnology Road map of the Kedah state was formulated under his supervision. He is currently the Chief Executive and Vice Chancellor of AIMST University, Kedah Darul Aman, Malaysia. He can be contacted at ravichandran@aimst.edu.my.

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